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(57) Abstract

An enzymatic nucleic acid molecule with RNA cleaving activity, wherein said nucleic acid molecule modulates the expression of a gene in a plant. A transgenic plant comprising nucleic acids encoding for an enzymatic nucleic acid molecule with RNA cleaving activity, wherein said nucleic acid molecule modulates the expression of a gene in said plant.

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DESCRIPTION

COMPOSITIONS AND METHOD FOR MODULATION OF GENE EXPRESSION IN PLANTS

This application is a continuation-in-part of: 1) a Non-Provisional application by Edington, entitled "Method for the production of transgenic plants deficient in starch granule bound glucose starch glycosyl transferase activity" filed on September 2, 1994 as U.S.S.N. 08/300,726; and 2) a Provisional application by Zwick *et al.*, entitled "Composition and method for modification of fatty acid saturation profile in plants" filed on July 13, 1995, as U.S.S.N 60/001,135. Both of these applications in their entirety, including drawings, are hereby incorporated by reference herein.

Background of the Invention

The present invention concerns compositions and methods for the modulation of gene expression in plants, specifically using enzymatic nucleic acid molecules.

The following is a brief description of regulation of gene expression in plants. The discussion is not meant to be complete and is provided only for understanding of the invention that follows. This summary is not an admission that any of the work described below is prior art to the claimed invention.

There are a variety of strategies for modulating gene expression in plants. Traditionally, antisense RNA (reviewed in Bourque, 1995 *Plant Sci* 105, 125-149) and co-suppression (reviewed in Jorgensen, 1995 *Science* 268, 686-691) approaches have been used to modulate gene expression. Insertion mutagenesis of genes have also been used to silence gene expression. This approach, however, cannot be designed to specifically inactivate the gene of interest. Applicant believes that ribozyme technology offers an attractive new means to alter gene expression in plants.

Naturally occurring antisense RNA was first discovered in bacteria over a decade ago (Simons and Kleckner, 1983 *Cell* 34, 683-691). It is thought to be one way in which bacteria can regulate their gene expression (Green et al., 1986 *Ann. Rev. Biochem.* 55: 567-597; Simons 1988 *Gene* 72: 35-44). The first demonstration of antisense-mediated inhibition of gene expression was reported in mammalian cells (Izant and Weintraub 1984 *Cell* 36: 1007-1015). There are many examples in the literature for the use of antisense RNA to modulate gene expression in plants. Following are a few examples:

Shewmaker *et al.*, U.S. Patent Nos. 5,107,065 and 5, 453,566 disclose methods for regulating gene expression in plants using antisense RNA.

It has been shown that an antisense gene expressed in plants can act as a dominant suppressor gene. Transgenic potato plants have been produced which express RNA antisense to potato or cassava granule bound starch synthase (GBSS). In both of these cases, transgenic plants have been constructed which have reduced or no GBSS activity or protein. These transgenic plants give rise to potatoes containing starch with dramatically reduced amylose levels (Visser *et al.* 1991, *Mol. Gen. Genet.* 225: 2889-296; Salehuzzaman *et al.* 1993, *Plant Mol. Biol.* 23: 947-962).

Kull *et al.*, 1995, *J. Genet. & Breed.* 49, 69-76 reported inhibition of amylose biosynthesis in tubers from transgenic potato lines mediated by the expression of antisense sequences of the gene for granule-bound starch synthase (GBSS). The authors, however, indicated a failure to see any *in vivo* activity of ribozymes targeted against the GBSS RNA.

Antisense RNA constructs targeted against Δ-9 desaturase enzyme in canola have been shown to increase the level of stearic acid (C18:0) from 2% to 40% (Knutzon et. al., 1992 *Proc. Natl. Acad. Sci.* 89, 2624). There was no decrease in total oil content or germination efficiency in one of the high stearate lines. Several recent reviews are available which illustrate the utility of plants with modified oil composition (Ohlrogge, J. B. 1994 *Plant Physiol.* 104, 821; Kinney, A. J. 1994 *Curr. Opin. Cell Biol.* 5, 144; Gibson et al. 1994 *Plant Cell Envir.* 17, 627).

Homologous transgene inactivation was first documented in plants as an unexpected result of inserting a transgene in the sense orientation and finding that both the gene and the transgene were down-regulated (Napoli et al., 1990 *Plant Cell* 2: 279-289). There appears to be at least two mechanisms for inactivation of homologous genetic sequences. One appears to be transcriptional inactivation via methylation, where duplicated DNA regions signal endogenous mechanisms for gene silencing. This approach of gene modulation involves either the introduction of multiple copies of transgenes or transformation of plants with transgenes with homology to the gene of interest (Ronchi et al 1995 *EMBO J.* 14: 5318-5328). The other mechanism of co-suppression is post-transcriptional, where the combined levels of expression from both the gene and the transgene is thought to produce high levels of transcript which triggers threshold-induced

degradation of both messages (van Bokland et al., 1994 *Plant J.* 6: 861-877). The exact molecular basis for co-suppression is unknown.

Unfortunately, b th antisense and co-suppression technologies are subject to problems in heritability of the desired trait (Finnegan and McElroy 1994 *Bio/Technology* 12: 883-888). Currently, there is no easy way to specifically inactivate a gene of interest at the DNA level in plants (Pazkowski et al., 1988 *EMBO J.* 7: 4021-4026). Transposon mutagenesis is inefficient and not a stable event, while chemical mutagenesis is highly non-specific.

Applicant believes that ribozymes present an attractive alternative and because of their catalytic mechanism of action, have advantages over competing technologies. However, there have been difficulties in demonstrating the effectiveness of ribozymes in modulating gene expression in plant systems (Mazzolini et al., 1992 *Plant Mol. Biol.* 20: 715-731; Kull et al., 1995 *J. Genet. & Breed.* 49: 69-76). Although there are reports in the literature of ribozyme activity in plants cells, almost all of them involve down regulation of exogenously introduced genes, such as reporter genes in transient assays (Steinecke et al., 1992 *EMBO J.* 11:1525-1530; Perriman et al., 1993 *Antisense Res. Dev.* 3: 253-263; Perriman *et al.*, 1995, *Proc. Natl. Acad. Sci. USA*, 92, 6165).

There are also several publications, [*e.g.*, Lamb and Hay, 1990, *J. Gen. Virol.* 71, 2257-2264; Gerlach *et al.*, International PCT Publication No. *WO 91/13994*; Xu *et al.*, 1992, *Science in China (Ser. B)* 35, 1434-1443; Edington and Nelson, 1992, in *Gene Regulation: Biology of antisense RNA and DNA*, eds. R. P. Erickson and J. G. Izant, pp 209-221, Raven Press, NY.; Atkins *et al.*, International PCT Publication No. *WO 94/00012*; Lenee *et al.*, International PCT Publication Nos. *WO 94/19476* and *WO 9503404*, Atkins *et al.*, 1995, *J. Gen. Virol.* 76, 1781-1790; Gruber *et al.*, 1994, *J. Cell. Biochem.* Suppl. 18A, 110 (X1-406) and Feyter *et al.*, 1996, *Mol. Gen. Genet.* 250, 329-338], that propose using hammerhead ribozymes to modulate: virus replication, expression of viral genes and/or reporter genes. None of these publications report the use of ribozymes to modulate the expression of plant genes.

Mazzolini *et al.*, 1992, *Plant. Mol. Bio.* 20, 715-731; Steinecke *et al.*, 1992, *EMBO. J.* 11, 1525-1530; Perriman *et al.*, 1995, *Proc. Natl. Acad. Sci.* USA., 92, 6175-6179; Wegener *et al.*, 1994, *Mol. Gen. Genet.* 245, 465-470; and Steinecke *et al.*, 1994, *Gene*, 149, 47-54, describe the use of hammerhead ribozymes to inhibit expression of reporter genes in plant cells.

Bennett and Cullimore, 1992 *Nucleic Acids Res.* 20, 831-837 demonstrate hammerhead ribozyme-mediated *in vitro* cleavage of *glna, glnb, glng and glnd* RNA, coding for glutamine synthetase enzyme in *Phaseolus vulgaris*.

Hitz *et al.*, (WO 91/18985) describe a method for using the soybean Δ-9 desaturase enzyme to modify plant oil composition. The application describes the use of soybean Δ-9 desaturase sequence to isolate Δ-9 desaturase genes from other species.

The references cited above are distinct from the presently claimed invention since they do not disclose and/or contemplate the use of ribozymes in maize. Furthermore, Applicant believes that the references do not disclose and/or enable the use of ribozymes to down regulate genes in plant cells, let alone plants.

Summary Of The Invention

The invention features modulation of gene expression in plants specifically using enzymatic nucleic acid molecules. Preferably, the gene is an endogenous gene. The enzymatic nucleic acid molecule with RNA cleaving activity may be in the form of, but not limited to, a hammerhead, hairpin, hepatitis delta virus, group I intron, group II intron, RNaseP RNA, *Neurospora VS* RNA and the like. The enzymatic nucleic acid molecule with RNA cleaving activity may be encoded as a monomer or a multimer, preferably a multimer. The nucleic acids encoding for the enzymatic nucleic acid molecule with RNA cleaving activity may be operably linked to an open reading frame. Gene expression in any plant species may be modified by transformation of the plant with the nucleic acid encoding the enzymatic nucleic acid molecules with RNA cleaving activity. There are also numerous technologies for transforming a plant: such technologies include but are not limited to transformation with *Agrobacterium*, bombarding with DNA coated microprojectiles, whiskers, or electroporation. Any target gene may be modified with the nucleic acids encoding the enzymatic nucleic acid molecules with RNA cleaving activity. Two targets which are exemplified herein are delta 9 desaturase and granule bound starch synthase (GBSS).

Until the discovery of the inventions herein, nucleic acid-based reagents, such as enzymatic nucleic acids (ribozymes), had yet to be demonstrated to modulate and/or inhibit gene expression in plants such as monocot plants (*e.g., corn)*. Ribozymes can be used to modulate a specific trait of a plant cell, for example, by modulating the activity of an enzyme involved in a biochemical pathway. It may be desirable, in some instances, to

decrease the level of expression of a particular gene, rather than shutting down expression completely: ribozymes can be used to achieve this. Enzymatic nucleic acid-based techniques were developed herein to allow directed modulation of gene expression to generate plant cells, plant tissues or plants with altered phenotype.

Ribozymes (*i.e.*, enzymatic nucleic acids) are nucleic acid molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript, and efficient cleavage has been achieved *in vitro* and *in vivo* (Zaug *et al.*, 1986, *Nature* 324, 429; Kim et al., 1987, *Proc. Natl. Acad. Sci. USA* 84, 8788; Dreyfus, 1988, *Einstein Quarterly J. Bio. Med.*, 6, 92; Hascloff and Gerlach, 1988, *Nature* 334 585; Cech, 1988, *JAMA* 260, 3030; Murphy and Cech, 1989, *Proc. Natl. Acad. Sci.* USA., 86, 9218; Jefferies et al., 1989, *Nucleic Acids Research* 17, 1371).

Because of their sequence-specificity, *trans*-cleaving ribozymes may be used as efficient tools to modulate gene expression in a variety of organisms including plants, animals and humans (Bennett *et al., supra*; Edington *et al., supra;* Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Ribozymes can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the mRNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a particular phenotype and/or disease state can be selectively inhibited.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Figure 1 is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥ 2 base-pairs long. Each N is any nucleotide and each • represents a base pair.

Figure 2a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 2b is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck (1987, *Nature*, 327, 596-600) into a substrate and enzyme portion; Figure 2c is a similar diagram showing the hammerhead divided by Haseloff and

Gerlach (1988, *Nature*, 334, 585-591) into two portions; and Figure 2d is a similar diagram showing the hammerhead divided by Jeffries and Symons (1989, *Nucl. Acids. Res.*, 17, 1371-1371) into two portions.

Figure 3 is a diagrammatic representation of the general structure of a hairpin ribozyme. Helix 2 (H2) is provided with a least 4 base pairs (*i.e.*, n is 1, 2, 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.*, m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.*, r is ≥ 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.*, 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.*. o and p is each independently from 0 to any number, *e.g.*, 20) as long as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.*, without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" is ≥ 2 bases. The connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "_____" refers to a covalent bond.

Figure 4 is a representation of the general structure of the hepatitis Δ virus ribozyme domain known in the art.

Figure 5 is a representation of the general structure of the self-cleaving VS RNA ribozyme domain.

Figure 6 is a schematic representation of an RNaseH accessibility assay. Specifically, the left side of Figure 6 is a diagram of complementary DNA oligonucleotides bound to accessible sites on the target RNA. Complementary DNA oligonucleotides are represented by broad lines labeled A, B, and C. Target RNA is represented by the thin, twisted line. The right side of Figure 6 is a schematic of a gel separation of uncut target RNA from a cleaved target RNA. Detection of target RNA is by autoradiography of body-labeled, T7 transcript. The bands common to each lane

represent uncleaved target RNA; the bands unique to each lane represent the cleaved products.

Figure 7 is a graphical representation of RNaseH accessibility of GBSS RNA.

Figure 8 is a graphical representation of GBSS RNA cleavage by ribozymes at different temperatures.

Figure 9 is a graphical representation of GBSS RNA cleavage by multiple ribozymes.

Figure 10 lists the nucleotide sequence of Δ-9 desaturase cDNA isolated from *Zea mays*.

Figures 11 and 12 are diagrammatic representations of fatty acid biosynthesis in plants. Figure 11 has been adapted from Gibson *et al.*, 1994, *Plant Cell Envir.* 17, 627.

Figures 13 and 14 are graphical representations of RNaseH accessibility of Δ-9 desaturase RNA.

Figure 15 shows cleavage of Δ-9 desaturase RNA by ribozymes *in vitro*. 10/10 represents the length of the binding arms of a hammerhead (HH) ribozyme. 10/10 means helix 1 and helix 3 each form 10 base-pairs with the target RNA (Fig. 1). 4/6 and 6/6, represent helix2/helix1 interaction between a hairpin ribozyme and its target. 4/6 means the hairpin (HP) ribozyme forms four base-paired helix 2 and a six base-paired helix 1 complex with the target (see Fig. 3). 6/6 means, the hairpin ribozyme forms a 6 base-paired helix 2 and a six base-paired helix 1 complex with the target. The cleavage reactions were carried out for 120 min at 26°C.

Figure 16 shows the effect of arm-length variation on the activity of HH and HP ribozymes *in vitro*. 7/7, 10/10 and 12/12 are essentially as described above for the HH ribozyme. 6/6, 6/8, 6/12 represents varying helix 1 length and a constant (6 bp) helix 2 for a hairpin ribozyme. The cleavage reactions were carried out essentially as described above.

Figures 17, 18, 19 and 23 are diagrammatic representations of non-limiting strategies to construct a transcript comprising multiple ribozyme motifs that are the same or different, targeting various sites within Δ-9 desaturase RNA.

Figures 20 and 21 show *in vitro* cleavage of Δ-9 desaturase RNA by ribozymes that are transcribed from DNA templates using bacteriophage T7 RNA polymerase enzyme.

Figure 22 diagrammatic representation of a non-limiting strategy to construct a transcript comprising multiple ribozyme motifs that are the same or different targeting various sites within GBSS RNA.

Figure 24 shows cleavage of Δ-9 desaturase RNA by ribozymes. 453 Multimer, represents a multimer ribozyme construct targeted against hammerhead ribozyme sites 453, 464, 475 and 484. 252 Multimer, represents a multimer ribozyme construct targeted against hammerhead ribozyme sites 252, 271, 313 and 326. 238 Multimer, represents a multimer ribozyme construct targeted against three hammerhead ribozyme sites 252, 259 and 271 and one hairpin ribozyme site 238 (HP). 259 Multimer, represents a multimer ribozyme construct targeted against two hammerhead ribozyme sites 271 and 313 and one hairpin ribozyme site 259 (HP).

Figure 25 illustrates GBSS mRNA levels in Ribozyme minus Controls (C, F, I, J, N, P, Q) and Active Ribozyme RPA63 Transformants (AA, DD, EE, FF, GG, HH, JJ, KK).

Figure 26 illustrates Δ9 desaturase mRNA levels in Non-transformed plants (NT), 85-06 High Stearate Plants (1, 3, 5, 8, 12, 14), and Transformed (irrelevant ribozyme) Control Plants (RPA63-33, RPA63-51, RPA63-65).

Figure 27 illustrates Δ9 desaturase mRNA levels in Non-transformed plants (NTO), 85-15 High Stearate Plants (01, 06, 07, 10, 11, 12), and 85-15 Normal Stearate Plants (02, 05, 09, 14).

Figure 28 illustrates Δ9 desaturase mRNA levels in Non-transformed plants (NTY), 113-06 Inactive Ribozyme Plants (02, 04, 07, 10,11).

Figures 29a and 29b illustrate Δ9 desaturase protein levels in maize leaves (R0). (a) Line HiII, plants a-e nontransformed and ribozyme inactive line RPA113-17, plants 1-6. (b) Ribozyme active line RPA85-15, plants 1-15.

Figure 30 illustrates stearic acid in leaves of RPA85-06 plants.

Figure 31 illustrates stearic acid in leaves of RPA85-15 plants, results of three assays.

Figure 32 illustrates stearic acid in leaves of RPA113-06 plants.

Figure 33 illustrates stearic acid in leaves of RPA113-17 plants.

Figure 34 illustrates stearic acid in leaves of control plants.

Figure 35 illustrates leaf stearate in R1 plants from a high stearate plant cross (RPA85-15.07 self).

Figure 36 illustrates Δ9 desaturase levels in next generation maize leaves (R1). * indicates those plants that showed a high stearate content.

Figure 37 illustrates stearic acid in individual somatic embryos from a culture (308/430-012) transformed with antisense Δ9 desaturase.

Figure 38 illustrates stearic acid in individual somatic embryos from a culture (308/430-015) transformed with antisense Δ9 desaturase.

Figure 39 illustrates stearic acid in individual leaves from plants regenerated from a culture (308/430-012) transformed with antisense Δ9 desaturase.

Figure 40 illustrates amylose content in a single kernel of untransformed control line (Q806 and antisense line 308/425-12.2.1.

Figure 41 illustrates GBSS activity in single kernels of a southern negative line (RPA63-0306) and Southern positive line RPA63-0218.

Figure 42 illustrates a transformation vector that can be used to express the enzymatic nucleic acid of the present invention.

Detailed Description Of The Invention

The present invention concerns compositions and methods for the modulation of gene expression in plants specifically using enzymatic nucleic acid molecules.

The following phrases and terms are defined below:

By "inhibit" or "modulate" is meant that the activity of enzymes such as GBSS and Δ-9 desaturase or level of mRNAs encoded by these genes is reduced below that observed in the absence of an enzymatic nucleic acid and preferably is below that level observed in

the presence of an inactive RNA molecule able to bind to the same site on the mRNA, but unable to cleave that RNA.

By "enzymatic nucleic acid molecule" it is meant a nucleic acid molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave that target. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave RNA (or DNA) and thereby inactivate a target RNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic nucleic acid molecule to the target RNA to allow the cleavage to occur. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, nucleozyme, DNAzyme, RNA enzyme, RNAzyme, polyribozymes, molecular scissors, self-splicing RNA, self-cleaving RNA, cis-cleaving RNA, autolytic RNA, endoribonuclease, minizyme, leadzyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The term encompasses enzymatic RNA molecule which include one or more ribonucleotides and may include a majority of other types of nucleotides or abasic moieties, as described below.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequences by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver and/or express a desired nucleic acid.

By "gene" is meant a nucleic acid that encodes an RNA.

By "plant gene" is meant a gene encoded by a plant.

By "endogenous" gene is meant a gene normally found in a plant cell in its natural location in the genome.

By "foreign" or "heterologous" gene is meant a gene not normally found in the host plant cell, but that is introduced by standard gene transfer techniques.

By "nucleic acid" is meant a molecule which can be single-stranded or double-stranded, composed of nucleotides containing a sugar, a phosphate and either a purine or pyrimidine base which may be same or different, and may be modified or unmodified.

By "genome" is meant genetic material contained in each cell of an organism and/or a virus.

By "mRNA" is meant RNA that can be translated into protein by a cell.

By "cDNA" is meant DNA that is complementary to and derived from a mRNA.

By "dsDNA" is meant a double stranded cDNA.

By "sense" RNA is meant RNA transcript that comprises the mRNA sequence.

By "antisense RNA" is meant an RNA transcript that comprises sequences complementary to all or part of a target RNA and/or mRNA and that blocks the expression of a target gene by interfering with the processing, transport and/or translation of its primary transcript and/or mRNA. The complementarity may exist with any part of the target RNA, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. Antisense RNA is normally a mirror image of the sense RNA.

By "expression", as used herein, is meant the transcription and stable accumulation of the enzymatic nucleic acid molecules, mRNA and/or the antisense RNA inside a plant cell. Expression of genes involves transcription of the gene and translation of the mRNA into precursor or mature proteins.

By "cosuppression" is meant the expression of a foreign gene, which has substantial homology to an gene, and in a plant cell causes the reduction in activity of the foreign and/or the endogenous protein product.

By "altered levels" is meant the level of production of a gene product in a transgenic organism is different from that of a normal or non-transgenic organism.

By "promoter" is meant nucleotide sequence element within a gene which controls the expression of that gene. Promoter sequence provides the recognition for RNA polymerase and other transcription factors required for efficient transcription. Promoters from a variety of sources can be used efficiently in plant cells to express ribozymes. For example, promoters of bacterial origin, such as the octopine synthetase promoter, the

nopaline synthase promoter, the manopine synthetase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S); plant promoters, such as the ribulose-1,6-biphosphate (RUBP) carboxylase small subunit (ssu), the beta-conglycinin promoter, the phaseolin promoter, the ADH promoter, heat-shock promoters, and tissue specific promoters. Promoter may also contain certain enhancer sequence elements that may improve the transcription efficiency.

By "enhancer" is meant nucleotide sequence element which can stimulate promoter activity (Adh).

By "constitutive promoter" is meant promoter element that directs continuous gene expression in all cells types and at all times (actin, ubiquitin, CaMV 35S).

By "tissue-specific" promoter is meant promoter element responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (zein, oleosin, napin, ACP).

By "development-specific" promoter is meant promoter element responsible for gene expression at specific plant developmental stage, such as in early or late embryogenesis.

By "inducible promoter" is meant promoter element which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; and stress.

By a "plant" is meant a photosynthetic organism, either eukaryotic and prokaryotic.

By "angiosperm" is meant a plant having its seed enclosed in an ovary (*e.g.*, coffee, tobacco, bean, pea).

By "gymnosperm" is meant a plant having its seed exposed and not enclosed in an ovary (*e.g.*, pine, spruce).

By "monocotyledon" is meant a plant characterized by the presence of only one seed leaf (primary leaf of the embryo). For example, maize, wheat, rice and others.

By "dicotyledon" is meant a plant producing seeds with two cotyledons (primary leaf of the embryo). For example, coffee, canola, peas and others.

By "transgenic plant" is meant a plant expressing a foreign gene.

By "open reading frame" is meant a nucleotide sequence, without introns, encoding an amino acid sequence, with a defined translation initiation and termination region.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a desired target. The enzymatic nucleic acid molecule may be targeted to a highly specific sequence region of a target such that specific gene inhibition can be achieved. Alternatively, enzymatic nucleic acid can be targeted to a highly conserved region of a gene family to inhibit gene expression of a family of related enzymes. The ribozymes can be expressed in plants that have been transformed with vectors which express the nucleic acid of the present invention.

The enzymatic nature of a ribozyme is advantageous over other technologies, since the concentration of ribozyme necessary to affect a therapeutic treatment is lower. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme.

Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of these ribozymes. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

In one of the preferred embodiments of the inventions herein, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the

motif of a hepatitis Δ virus, group I intron, group II intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi *et al.*, 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; of the hepatitis Δ virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNaseP motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; and of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

The enzymatic nucleic acid molecules of the instant invention will be expressed within cells from eukaryotic promoters [*e.g.*, Gerlach *et al.*, International PCT Publication No. *WO 91/13994*; Edington and Nelson, 1992, in *Gene Regulation: Biology of Antisense RNA and DNA*, eds. R. P. Erickson and J. G. Izant, pp 209-221, Raven Press, NY.; Atkins *et al.*, International PCT Publication No. *WO 94/00012*; Lenee *et al.*, International PCT Publication Nos. *WO 94/19476* and *WO 9503404*, Atkins *et al.*, 1995, *J. Gen. Virol.* 76, 1781-1790; McElroy and Brettell, 1994, *TIBTECH* 12, 62; Gruber *et al.*, 1994, *J. Cell. Biochem.* Suppl. 18A, 110 (X1-406)and Feyter *et al.*, 1996, *Mol. Gen. Genet.* 250, 329-338; all of these are incorporated by reference herein]. Those skilled in the art will realize from the teachings herein that any ribozyme can be expressed in eukaryotic plant cells from an appropriate promoter. The ribozymes expression is under the control of a constitutive promoter, a tissue-specific promoter or an inducible promoter.

To obtain the ribozyme mediated modulation, the ribozyme RNA is introduced into the plant. Although examples are provided below for the construction of the plasmids used in the transformation experiments illustrated herein, it is well within the skill of an

artisan to design numerous different types of plasmids which can be used in the transformation of plants._see Bevan, 1984 , *Nucl. Acids Res.* 12, 8711-8721, which is incorporated by reference. There are also numerous ways to transform plants. In the examples below embryogenic maize cultures were helium blasted. In addition to using the gene gun (US Patents 4,945,050 to Cornell and 5,141,131 to DowElanco), plants may be transformed using *Agrobacterium* technology, see US Patent 5,177,010 to University of Toledo, 5,104,310 to Texas A&M, European Patent Application 0131624B1, European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot, US Patents 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot, European Patent Applications 116718, 290799, 320500 all to MaxPlanck, European Patent Applications 604662 and 627752 to Japan Tobacco, European Patent Applications 0267159, and 0292435 and US Patent 5,231,019 all to Ciba Geigy, US Patents 5,463,174 and 4,762,785 both to Calgene, and US Patents 5,004,863 and 5,159,135 both to Agracetus; whiskers technology, see US Patents 5,302,523 and 5,464,765 both to Zeneca; electroporation technology, see WO 87/06614 to Boyce Thompson Institute, 5,472,869 and 5,384,253 both to Dekalb, WO9209696 and WO9321335 both to PGS; all of which are incorporated by reference herein in totality. In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign material (typically plasmids containing RNA or DNA) may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue type I and II, and any tissue which is receptive to transformation and subsequent regeneration into a transgenic plant. Another variable is the choice of a selectable marker. The preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to chlorosulfuron, hygromyacin, PAT and/or bar, bromoxynil, kanamycin and the like. The bar gene may be isolated from *Strptomuces*, particularly from the *hygroscopicus* or *viridochromogenes* species. The bar gene codes for phosphinothricin acetyl transferase (PAT) that inactivates the active ingradient in the herbicide bialaphos phosphinothricin (PPT). Thus, numerous combinations of technologies may be used in employing ribozyme mediated modulation.

The ribozymes may be expressed individually as monomers, *i.e.*, one ribozyme targeted against one site is expressed per transcript. Alternatively, two or more ribozymes targeted against more than one target site are expressed as part of a single RNA transcript. A single RNA transcript comprising more than one ribozyme targeted against

more than one cleavage site are readily generated to achieve efficient modulation of gene expression. Ribozymes within these multimer constructs are the same or different. For example, the multimer construct may comprise a plurality of hammerhead ribozymes or hairpin ribozymes or other ribozyme motifs. Alternatively, the multimer construct may be designed to include a plurality of different ribozyme motifs, such as hammerhead and hairpin ribozymes. More specifically, multimer ribozyme constructs are designed, wherein a series of ribozyme motifs are linked together in tandem in a single RNA transcript. The ribozymes are linked to each other by nucleotide linker sequence, wherein the linker sequence may or may not be complementary to the target RNA. Multimer ribozyme constructs (polyribozymes) are likely to improve the effectiveness of ribozyme-mediated modulation of gene expression.

The activity of ribozymes can also be augmented by their release from the primary transcript by a second ribozyme (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595, both hereby incorporated in their totality by reference herein; Ohkawa, J., et al., 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira, K., *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura, M., *et al.*, 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994 *J. Biol. Chem*. 269, 25856).

Ribozyme-mediated modulation of gene expression can be practiced in a wide variety of plants including angiosperms, gymnosperms, monocotyledons, and dicotyledons. Plants of interest include but are not limited to: cereals, such as rice, wheat, barley, maize; oil-producing crops, such as soybean, canola, sunflower, cotton, maize, cocoa, safflower, oil palm, coconut palm, flax, castor, peanut; plantation crops, such as coffee and tea; fruits, such as pineapple, papaya, mango, banana, grapes, oranges, apples; vegetables, such as cauliflower, cabbage, melon, green pepper, tomatoes, carrots, lettuce, celery, potatoes, broccoli; legumes, such as soybean, beans, peas; flowers, such as carnations, chrysanthemum, daisy, tulip, gypsophila, alstromeria, marigold, petunia, rose; trees such as olive, cork, poplar, pine; nuts, such as walnut, pistachio, and others. Following are a few non-limiting examples that describe the general utility of ribozymes in modulation of gene expression.

Ribozyme-mediated down regulation of the expression of genes involved in caffeine synthesis can be used to significantly change caffeine concentration in coffee beans. Expression of genes, such as 7-methylxanthosine and/or 3-methyl transferase in coffee plants can be readily modulated using ribozymes to decrease caffeine synthesis (Adams and Zarowitz, *US Patent No. 5,334,529*; incorporated by reference herein).

Transgenic tobacco plants expressing ribozymes targeted against genes involved in nicotine production, such as N-methylputrescine oxidase or putrescine N-methyl transferase (Shewmaker *et al., supra*), would produce leaves with altered nicotine concentration.

Transgenic plants expressing ribozymes targeted against genes involved in ripening of fruits, such as ethylene-forming enzyme, pectin methyltransferase, pectin esterase, polygalacturonase, 1-aminocyclopropane carboxylic acid (ACC) synthase, ACC oxidase genes (Smith *et al.*, 1988, *Nature*, 334, 724; Gray *et al.*, 1992, *Pl. Mol. Biol.*, 19, 69; Tieman *et al.*, 1992, *Plant Cell*, 4, 667; Picton *et al.*, 1993, *The Plant J.* 3, 469; Shewmaker *et al., supra*; James *et al.*, 1996, *Bio/Technology*, 14, 56), would delay the ripening of fruits, such as tomato and apple.

Transgenic plants expressing ribozymes targeted against genes involved in flower pigmentation, such as chalcone synthase (CHS), chalcone flavanone isomerase (CHI), phenylalanine ammonia lyase, or dehydroflavonol (DF) hydroxylases, DF reductase (Krol van der, *et al.*, 1988, *Nature*, 333, 866; Krol van der, *et al.*, 1990, *Pl. Mol. Biol.*, 14, 457; Shewmaker *et al., supra*; Jorgensen, 1996, *Science*, 268, 686), would produce flowers, such as roses, petunia, with altered colors.

Lignins are organic compounds essential for maintaining mechanical strength of cell walls in plants. Although essential, lignins have some disadvantages. They cause indigestibility of sillage crops and are undesirable to paper production from wood pulp and others. Transgenic plants expressing ribozymes targeted against genes involved in lignin production such as, *O*-methyltransferase, cinnamoyl-CoA:NADPH reductase or cinnamoyl alcohol dehydrogenase (Doorsselaere *et al.*, 1995, *The Plant J.* 8, 855; Atanassova *et al.*, 1995, *The Plant J.* 8, 465; Shewmaker *et al., supra*; Dwivedi *et al.*, 1994, *Pl. Mol. Biol.*, 26, 61), would have altered levels of lignin.

Other useful targets for useful ribozymes are disclosed in Draper *et al.*, International PCT Publication No. *WO 93/23569*, Sullivan *et al.*, International PCT Publication No. *WO 94/02595*, as well as by Stinchcomb *et al.*, International PCT Publication No. *WO 95/31541*, and hereby incorporated by reference herein in totality.

<u>Modulation of granule bound starch synthase gene expression in plants:</u>

In plants, starch biosynthesis occurs in both chloroplasts (short term starch storage) and in the amyloplast (long term starch storage). Starch granules normally

consist of a linear chain of α(1-4)-linked α-D-glucose units (amylose) and a branched form of amylose cross-linked by α(1-6) bonds (amylopectin). An enzyme involved in the synthesis of starch in plants is starch synthase which produces linear chains of α (1-4)-glucose using ADP-glucose. Two main forms of starch synthase are found in plants: granule bound starch synthase (GBSS) and a soluble form located in the stroma of chloroplasts and in amyloplasts (soluble starch synthase). Both forms of this enzyme utilize ADP-D-glucose while the granular bound form also utilizes UDP-D-glucose, with a preference for the former. The GBSS, known as waxy protein, has a molecular mass of between 55 to about 70 kDa in a variety of plants in which it has been characterized. Mutations affecting the GBSS gene in several plant species has resulted in the loss of amylose, while the total amount of starch has remained relatively unchanged. In addition to a loss of GBSS activity, these mutants also contain altered, reduced levels, or no GBSS protein (Mac Donald and Preiss, Plant Physiol. 78: 849-852 (1985), Sano, Theor. Appl. Genet. 68: 467-473 (1984), Hovenkamp-Hermelink et al. Theor. Appl. Genet. 75: 217-221 91987), Shure et al. Cell 35, 225-233 (1983), Echt and Schwartz Genetics 99: 275-284 (1981)). The presence of a branching enzyme as well as a soluble ADP-glucose starch glycosyl transferase in both GBSS mutants and wild type plants indicates the existence of independent pathways for the formation of the branched chain polymer amylopectin and the straight chain polymer amylose.

The Wx (waxy) locus encodes a granule bound glucosyl transferase involved in starch biosynthesis. Expression of this enzyme is limited to endosperm, pollen and the embryo sac in maize. Mutations in this locus have been termed waxy due to the appearance of mutant kernels, which is the phenotype resulting from an reduction in amylose composition in the kernels. In maize, this enzyme is transported into the amyloplast of the developing endosperm where it catalyses production of amylose. Corn kernels are about 70% starch, of which 27% is linear amylose and 73% is amylopectin. Waxy is a recessive mutation in the gene encoding granule bound starch synthase (GBSS). Plants homozygous for this recessive mutation produce kernels that contain 100% of their starch in the form of amylopectin.

Ribozymes, with their catalytic activity and increased site specificity (as described below), represent more potent and perhaps more specific inhibitory molecules than antisense oligonucleotides. Moreover, these ribozymes are able to inhibit GBSS activity and the catalytic activity of the ribozymes is required for their inhibitory effect. For those of ordinary skill in the art, it is clear from the examples that other ribozymes may

be designed that cleave target mRNAs required for GBSS activity in plant species other than maize.

Thus, in a preferred embodiment, the invention features ribozymes that inhibit enzymes involved in amylose production, *e.g.*, by reducing GBSS activity. These endogenously expressed RNA molecules contain substrate binding domains that bind to accessible regions of the target mRNA. The RNA molecules also contain domains that catalyze the cleavage of RNA. The RNA molecules are preferably ribozymes of the hammerhead or hairpin motif. Upon binding, the ribozymes cleave the target mRNAs, preventing translation and protein accumulation. In the absence of the expression of the target gene, amylose production is reduced or inhibited. Specific examples are provided below *infra*.

Preferred embodiments include the ribozymes having binding arms which are complementary to the binding sequences in Tables IIIA, VA and VB. Examples of such ribozymes are shown in Tables IIIB - V. Those in the art will recognize that while such examples are designed to one plant's (*e.g.*, maize) mRNA, similar ribozymes can be made complementary to other plant species' mRNA. By complementary is thus meant that the binding arms enable ribozymes to interact with the target RNA in a sequence-specific manner to cause cleavage of a plant mRNA target. Examples of such ribozymes consist essentially of sequences shown in Tables IIIB - V.

Preferred embodiments are the ribozymes and methods for their use in the inhibition of starch granule bound ADP (UDP)-glucose: α-1,4-*D*-glucan 4-α-glucosyl transferase *i.e.*, granule bound starch synthase (GBSS) activity in plants. This is accomplished through the inhibition of genetic expression, with ribozymes, which results in the reduction or elimination of GBSS activity in plants.

In another aspect of the invention, ribozymes that cleave target molecules and inhibit amylose production are expressed from transcription units inserted into the plant genome. Preferably, the recombinant vectors capable of stable integration into the plant genome and selection of transformed plant lines expressing the ribozymes are expressed either by constitutive or inducible promoters in the plant cells. Once expressed, the ribozymes cleave their target mRNAs and reduce amylose production of their host cells. The ribozymes expressed in plant cells are under the control of a constitutive promoter, a tissue-specific promoter or an inducible promoter.

Modification of corn starch is an important application of ribozyme technology which is capable of reducing specific gene expression. A high level of amylopectin is desirable for the wet milling process of corn and there is also some evidence that high amylopectin corn leads to increased digestibility and therefore energy availability in feed. Nearly 10% of wet-milled corn has the waxy phenotype, but because of its recessive nature the traditional waxy varieties are very difficult for the grower to handle. Ribozymes targeted to cleave the GBSS mRNA and thus reduce GBSS activity in plants, and in particular, corn endosperm will act as a dominant trait and produce corn plants with the waxy phenotype that will be easier for the grower to handle.

Modification of fatty acid saturation profile in plants:

Fatty acid biosynthesis in plant tissues is initiated in the chloroplast. Fatty acids are synthesized as thioesters of acyl carrier protein (ACP) by the fatty acid synthase complex (FAS). Fatty acids with chain lengths of 16 carbons follow one of three paths: 1) they are released, immediately after synthesis, and transferred to glycerol 3-phosphate (G3P) by a chloroplast acyl transferase for further modification within the chloroplast; 2) they are released and transferred to Co-enzyme A (CoA) upon export from the plastid by thioesterases; or 3) they are further elongated to C18 chain lengths. The C18 chains are rapidly desaturated at the C9 position by stearoyl-ACP desaturase. This is followed by immediate transfer of the oleic acid (18:1) group to G3P within the chloroplast, or by export from the chloroplast and conversion to oleoyl-CoA by thioesterases (Somerville and Browse, 1991 *Science* 252: 80-87). The majority of C16 fatty acids follow the third pathway.

In corn seed oil the predominant triglycerides are produced in the endoplasmic reticulum. Most oleic acids (18:1) and some palmitic acids (16:0) are transferred to G3P from phosphatidic acids, which are then converted to diacyl glycerides and phosphatidyl choline. Further desaturation of the acyl chains on phosphatidyl choline by membrane bound desaturases takes place in the endoplasmic reticulum. Di- and tri-unsaturated chains are then released into the acyl-CoA pool and transferred to the C3 position of the glycerol backbone in diacyl glycerol in the production of triglycerides (Frentzen, 1993 in *Lipid Metabolism in Plants.*, p.195-230, (ed. Moore,T.S.) CRC Press, Boca Raton, FA.). A schematic of the plant fatty acid biosynthesis pathway is shown in Figures 11 and 12. The three predominant fatty acids in corn seed oil are linoleic acid (18:2, ~59%), oleic acid (18:1, ~26%), and palmitic acid (16:0, ~11%). These are average values and may be somewhat different depending on the genotype; however, composite samples of US Corn

Belt produced oil analyzed over the past ten years have consistently had this composition (Glover and Mertz, 1987 in: Nutritional Quality of Cereal Grains: genetic and agronomic improvement., p.183-336, (eds. Olson, R.A. and Frey, K.J.) Am. Soc. Agronomy. Inc. Madison, WI.; Fitch-Haumann, 1985 *J. Am. Oil Chem. Soc.* 62: 1524-1531; Strecker et al., 1990 in Edible fats and oils processing: basic principles and modern practices (ed. Erickson, D.R.) Am. Oil Chemists Soc. Champaign, IL.). This predominance of C18 chain lengths may reflect the abundance and activity of several key enzymes, such as the fatty acid synthase responsible for production of C18 carbon chains, the stearoyl-ACP desaturase (Δ-9 desaturase) for production of 18:1 and a microsomal Δ-12 desaturase for conversion of 18:1 to 18:2.

Δ-9 desaturase (also called stearoyl-ACP desaturase) of plants is a soluble chloroplast enzyme which uses C18 and occasionally C16-acyl chains linked to acyl carrier protein (ACP) as a substrate (McKeon, T.A. and Stumpf, P.K., 1982 *J. Biol. Chem.* 257: 12141-12147). This contrasts to the mammalian, lower eukaryotic and cyanobacterial Δ-9 desaturases. Rat and yeast Δ-9 desaturases are membrane bound microsomal enzymes using acyl-CoA chains as substrates, whereas cyanobacterial Δ-9 desaturase uses acyl chains on diacyl glycerol as substrate. To date several Δ-9 desaturase cDNA clones from dicotelydenous plants have been isolated and characterized (Shanklin and Somerville, 1991 *Proc. Natl. Acad. Sci. USA* 88: 2510-2514; Knutzon et al., 1991 *Plant Physiol.* 96: 344-345; Sato et al., 1992 *Plant Physiol.* 99: 362-363; Shanklin et al., 1991 *Plant Physiol.* 97: 467-468; Slocombe et al., 1992 *Plant. Mol. Biol.* 20: 151-155; Taylor et al., 1992 *Plant Physiol.* 100: 533-534; Thompson et al., 1991 *Proc. Natl. Acad. Sci. USA* 88: 2578-2582). Comparison of the different plant Δ-9 desaturase sequences suggests that this is a highly conserved enzyme, with high levels of identity both at the amino acid level (~90%) and at the nucleotide level (~80%). However, as might be expected from its very different physical and enzymological properties, no sequence similarity exists between plant and other Δ-9 desaturases (Shanklin and Somerville, *supra*).

Purification and characterization of the castor bean desaturase (and others) indicates that the Δ-9 desaturase is active as a homodimer; the subunit molecular weight is ~ 41 kDa. The enzyme requires molecular oxygen, NADPH, NADPH ferredoxin oxidoreductase and ferredoxin for activity *in vitro*. Fox *et al.*, 1993 (*Proc. Natl. Acad. Sci. USA* 90: 2486-2490) showed that upon expression in *E. coli* the castor bean enzyme contains four catalytically active ferrous atoms per homodimer. The oxidized enzyme

contains two identical diferric clusters, which in the presence of dithionite are reduced to the diferrous state. In the presence of stearoyl-CoA and O_2 the clusters return to the diferric state. This suggests that the desaturase belongs to a group of O_2 activating proteins containing diiron-oxo clusters. Other members of this group are ribonucleotide reductase and methane monooxygenase hydroxylase. Comparison of the predicted primary structure for these catalytically diverse proteins shows that all contain a conserved pair of amino acid sequences (Asp/Glu)-Glu-Xaa-Arg-His separated by ~80-100 amino acids.

Traditional plant breeding programs have shown that increased stearate levels can be achieved without deleterious consequences to the plant. In safflower (Ladd and Knowles, 1970 *Crop Sci.* 10: 525-527) and in soybean (Hammond and Fehr, 1984 *J. Amer. Oil Chem. Soc.* 61: 1713-1716; Graef *et al.*, 1985 *Crop Sci.* 25: 1076-1079) stearate levels have been increased significantly. This demonstrates the flexibility in fatty acid composition of seed oil.

Increases in Δ-9 desaturase activity have been achieved by the transformation of tobacco with the Δ-9 desaturase genes from yeast (Polashock et al., 1992 *Plant Physiol.* 100, 894) or rat (Grayburn et. al., 1992 *BioTechnology* 10, 675). Both sets of transgenic plants had significant changes in fatty acid composition, yet were phenotypically identical to control plants.

Corn (maize) has been used minimally for the production of margarine products because it has traditionally not been utilized as an oil crop and because of the relatively low seed oil content when compared with soybean and canola. However, corn oil has low levels of linolenic acid (18:3) and relatively high levels of palmitic (16:0) acid (desirable in margarine). Applicant believes that reduction in oleic and linoleic acid levels by down-regulation of Δ-9 desaturase activity will make corn a viable alternative to soybean and canola in the saturated oil market.

Margarine and confectionary fats are produced by chemical hydrogenation of oil from plants such as soybean. This process adds cost to the production of the margarine and also causes both *cis* and *trans* isomers of the fatty acids. *Trans* isomers are not naturally found in plant derived oils and have raised a concern for potential health risks. Applicant believes that one way to eliminate the need for chemical hydrogenation is to genetically engineer the plants so that desaturation enzymes are down-regulated. Δ-9

desaturase introduces the first double bond into 18 carbon fatty acids and is the key step effecting the extent of desaturation of fatty acids.

Thus, in a preferred embodiment, the invention concerns compositions (and methods for their use) for the modification of fatty acid composition in plants. This is accomplished through the inhibition of genetic expression, with ribozymes, antisense nucleic acid, cosuppression or triplex DNA, which results in the reduction or elimination of certain enzyme activities in plants, such as Δ-9 desaturase. Such activity is reduced in monocotyledon plants, such as maize, wheat, rice, palm, coconut and others. Δ-9 desaturase activity may also be reduced in dicotyledon plants such as sunflower, safflower, cotton, peanut, olive, sesame, cuphea, flax, jojoba, grape and others.

Thus, in one aspect, the invention features ribozymes that inhibit enzymes involved in fatty acid unsaturation, *e.g.*, by reducing Δ-9 desaturase activity. These endogenously expressed RNA molecules contain substrate binding domains that bind to accessible regions of the target mRNA. The RNA molecules also contain domains that catalyze the cleavage of RNA. The RNA molecules are preferably ribozymes of the hammerhead or hairpin motif. Upon binding, the ribozymes cleave the target mRNAs, preventing translation and protein accumulation. In the absence of the expression of the target gene, stearate levels are increased and unsaturated fatty acid production is reduced or inhibited. Specific examples are provided below in the Tables listed directly below.

In preferred embodiments, the ribozymes have binding arms which are complementary to the sequences in the Tables VI and VIII. Those in the art will recognize that while such examples are designed to one plant's (*e.g.*, corn) mRNA, similar ribozymes can be made complementary to other plant's mRNA. By complementary is thus meant that the binding arms of the ribozymes are able to interact with the target RNA in a sequence-specific manner and enable the ribozyme to cause cleavage of a plant mRNA target. Examples of such ribozymes are typically sequences defined in Tables VII and VIII. The active ribozyme typically contains an enzymatic center equivalent to those in the examples, and binding arms able to bind plant mRNA such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such binding and/or cleavage.

The sequences of the ribozymes that are particularly useful in this study, are shown in Tables VII and VIII.

Those in the art will recognize that ribozyme sequences listed in the Tables are representative only of many more such sequences where the enzymatic portion of the ribozyme (all but the binding arms) is altered to affect activity. For example, stem-loop II sequence of hammerhead ribozymes listed in Table IV (5'-GGCGAAAGCC-3') can be altered (substitution, deletion, and/or insertion) to contain any sequences, preferably provided that a minimum of a two base-paired stem structure can form. Similarly, stem-loop IV sequence of hairpin ribozymes listed in Tables V and VIII (5'-CACGUUGUG-3') can be altered (substitution, deletion, and/or insertion) to contain any sequence, preferably provided that a minimum of a two base-paired stem structure can form. Such ribozymes are equivalent to the ribozymes described specifically in the Tables.

In another aspect of the invention, ribozymes that cleave target molecules and reduce unsaturated fatty acid content in plants are expressed from transcription units inserted into the plant genome. Preferably, the recombinant vectors capable of stable integration into the plant genome and selection of transformed plant lines expressing the ribozymes are expressed either by constitutive or inducible promoters in the plant cells. Once expressed, the ribozymes cleave their target mRNAs and reduce unsaturated fatty acid production of their host cells. The ribozymes expressed in plant cells are under the control of a constitutive promoter, a tissue-specific promoter or an inducible promoter.

Modification of fatty acid profile is an important application of nucleic acid-based technologies which are capable of reducing specific gene expression. A high level of saturated fatty acid is desirable in plants that produce oils of commercial importance.

In a related aspect, this invention features the isolation of the cDNA sequence encoding Δ-9 desaturase in maize.

In preferred embodiments, hairpin and hammerhead ribozymes that cleave Δ-9 desaturase mRNA are also described. Those of ordinary skill in the art will understand from the examples described below that other ribozymes that cleave target mRNAs required for Δ-9 desaturase activity may now be readily designed and are within the scope of the invention.

While specific examples to corn RNA are provided, those in the art will recognize that the teachings are not limited to corn. Furthermore, the same target may be used in other plant species. The complementary arms suitable for targeting the specific plant RNA sequences are utilized in the ribozyme targeted to that specific RNA. The examples

and teachings herein are meant to be non-limiting, and those skilled in the art will recognize that similar embodiments can be readily generated in a variety of different plants to modulate expression of a variety of different genes, using the teachings herein, and are within the scope of the inventions.

Standard molecular biology techniques were followed in the examples herein. Additional information may be found in Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), Molecular Cloning a Laboratory Manual, second edition, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, which is incorporated herein by reference.

Examples

Example 1: Isolation of Δ 9 desaturase cDNA from *Zea mays*

Degenerate PCR primers were designed and synthesized to two conserved peptides involved in diiron-oxo group binding of plant Δ-9 desaturases. A 276 bp DNA fragment was PCR amplified from maize embryo cDNA and was cloned in to a vector. The predicted amino acid sequence of this fragment was similar to the sequence of the region separated by the two conserved peptides of dicot Δ-9 desaturase proteins. This was used to screen a maize embryo cDNA library. A total of 16 clones were isolated; further restriction mapping and hybridization identified one clone which was sequenced. Features of the cDNA insert are: a 1621 nt cDNA; 145 nt 5' and 294 nt 3' untranslated regions including a 18 nt poly A tail; a 394 amino acid open reading frame encoding a 44.7 kD polypeptide; and 85% amino acid identity with castor bean Δ-9 desaturase gene for the predicted mature protein. The complete sequence is presented in Figure 10.

Example 2: Identification of Potential Ribozyme Cleavage Sites for Δ9 desaturase

Approximately two hundred and fifty HH ribozyme sites and approximately forty three HP sites were identified in the corn Δ-9 desaturase mRNA. A HH site consists of a uridine and any nucleotide except guanosine (UH). Tables VI and VIII have a list of HH and HP ribozyme cleavage sites. The numbering system starts with 1 at the 5' end of a Δ-9 desaturase cDNA clone having the sequence shown in Fig. 10.

Ribozymes, such as those listed in Tables VII and VIII, can be readily designed and synthesized to such cleavage sites with between 5 and 100 or more bases as substrate binding arms (see Figs. 1 - 5). These substrate binding arms within a ribozyme allow the ribozyme to interact with their target in a sequence-specific manner.

Example 3: Selection of Ribozyme Cleavage Sites for Δ9 desaturase

The secondary structure of Δ-9 desaturase mRNA was assessed by computer analysis using algorithms, such as those developed by M. Zuker (Zuker, M., 1989 *Science*, 244, 48-52). Regions of the mRNA that did not form secondary folding structures with RNA/RNA stems of over eight nucleotides and contained potential hammerhead ribozyme cleavage sites were identified.

These sites were assessed for oligonucleotide accessibility by RNase H assays (see Example 4 *infra*).

Example 4: RNaseH Assays for Δ9 desaturase

Forty nine DNA oligonucleotides, each twenty one nucleotides long were used in RNase H assays. These oligonucleotides covered 108 sites within Δ-9 desaturase RNA. RNase H assays (Fig. 6) were performed using a full length transcript of the Δ-9 desaturase cDNA. RNA was screened for accessible cleavage sites by the method described generally in Draper *et al., supra*. Briefly, DNA oligonucleotides representing ribozyme cleavage sites were synthesized. A polymerase chain reaction was used to generate a substrate for T7 RNA polymerase transcription from corn cDNA clones. Labeled RNA transcripts were synthesized *in vitro* from these templates. The oligonucleotides and the labeled transcripts were annealed, RNAseH was added and the mixtures were incubated for 10 minutes at 37°C. Reactions were stopped and RNA separated on sequencing polyacrylamide gels. The percentage of the substrate cleaved was determined by autoradiographic quantitation using a Molecular Dynamics phosphor imaging system (Figs. 13 and 14).

Example 5: Hammerhead and Hairpin Ribozymes for Δ9 desaturase

Hammerhead (HH) and hairpin (HP) ribozymes were designed to the sites covered by the oligos which cleaved best in the RNase H assays. These ribozymes were then subjected to analysis by computer folding and the ribozymes that had significant secondary structure were rejected.

The ribozymes were chemically synthesized. The general procedures for RNA synthesis have been described previously (Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845-7854 and in Scaringe et al., 1990, *Nucl. Acids Res.*, 18, 5433-5341; Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677). Small scale syntheses were conducted on a 394

Applied Biosystems, Inc. synthesizer using a modified 2.5 μmol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-*O*-methylated nucleotides. Table II outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 μL of 0.1 M = 16.3 μmol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238 μL of 0.25 M = 59.5 μmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394, determined by colorimetric quantitation of the trityl fractions, was 97.5-99%. Other oligonucleotide synthesis reagents for the 394: Detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-Methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

Deprotection of the RNA was performed as follows. The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4 mL glass screw top vial and suspended in a solution of methylamine (MA) at 65°C for 10 min. After cooling to -20°C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H_2O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder.

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250 μL of a solution of 1.5 mL *N*-methylpyrrolidinone, 750 μL TEA and 1.0 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white powder.

Inactive hammerhead ribozymes were synthesized by substituting a U for G_5 and a U for A_{14} (numbering from (Hertel, K. J., *et al.*, 1992, *Nucleic Acids Res.*, 20, 3252).

The hairpin ribozymes were synthesized as described above for the hammerhead RNAs.

Ribozymes were also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). Ribozymes were purified by gel electrophoresis using general methods or were purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, 1996, *supra*, the totality of which is hereby incorporated herein by reference) and were resuspended in water. The sequences of the chemically synthesized ribozymes used in this study are shown below in Tables VII and VIII.

Example 6: Long substrate tests for Δ9 desaturase ribozymes

Target RNA used in this study was 1621 nt long and contained cleavage sites for all the HH and HP ribozymes targeted against Δ-9 desaturase RNA. A template containing T7 RNA polymerase promoter upstream of Δ-9 desaturase target sequence, was PCR amplified from a cDNA clone. Target RNA was transcribed from this PCR amplified template using T7 RNA polymerase. The transcript was internally labeled during transcription by including [α-^{32}P] CTP as one of the four ribonucleotide triphosphates. The transcription mixture was treated with DNase-I, following transcription at 37°C for 2 hours, to digest away the DNA template used in the transcription. The transcription mixture was resolved on a denaturing polyacrylamide gel. Bands corresponding to full-length RNA was isolated from a gel slice and the RNA was precipitated with isopropanol and the pellet was stored at 4°C.

Ribozyme cleavage reactions were carried out under ribozyme excess (k_{cat}/K_M) conditions (Herschlag and Cech, 1990, *Biochemistry* 29, 10159-10171). Briefly, 1 mM ribozyme and < 10 nM internally labeled target RNA were denatured separately by heating to 65°C for 2 min in the presence of 50 mM Tris.HCl, pH 7.5 and 10 mM $MgCl_2$. The RNAs were renatured by cooling to the reaction temperature (37°C, 26°C or 20°C) for 10-20 min. Cleavage reaction was initiated by mixing the ribozyme and target RNA at appropriate reaction temperatures. Aliquots were taken at regular intervals of time and the reaction was quenched by adding equal volume of stop buffer. The samples were resolved on 4 % sequencing gel.

The results from ribozyme cleavage reactions, at 26°C or 20°C, are summarized in Table IX and Figures 15 and 16. Of the ribozymes tested, seven hammerheads and two

hairpins showed significant cleavage of Δ-9 desaturase RNA (Figures 15 and 16). Ribozymes to other sites showed varied levels of activity.

Example 7: Cleavage of the target RNA using multiple ribozyme combinations for Δ9 desaturase

Several of the above ribozymes were incorporated into a multimer ribozyme construct which contains two or more ribozymes embedded in a contiguous stretch of complementary RNA sequence. Non-limiting examples of multimer ribozymes are shown in Figures 17, 18, 19 and 23. The ribozymes were made by annealling complementary oligonucleotides and cloning into an expression vector containing the Cauliflower Mosaic Virus 35S enhanced promoter (Franck *et al.*, 1985 *Cell* 21, 285), the maize Adh 1 intron (Dennis *et al.*, 1984 *Nucl. Acids Res.* 12, 3983) and the Nos polyadenylation signal (DePicker *et al.*, 1982 *J. Molec. Appl. Genet.* 1, 561). Cleavage assays with T7 transcripts made from these multimer-containing transcription units are shown in Figures 20 and 21. These are non-limiting examples; those skilled in the art will recognize that similar embodiments, consisting of other ribozyme combinations, introns and promoter elements, can be readily generated using techniques known in the art and are within the scope of this invention.

Example 8: Construction of Ribozyme expressing transcription units for Δ9 desaturase

Ribozymes targeted to cleave Δ-9 desaturase mRNA are endogenously expressed in plants, either from genes inserted into the plant genome (stable transformation) or from episomal transcription units (transient expression) which are part of plasmid vectors or viral sequences. These ribozymes can be expressed *via* RNA polymerase I, II, or III plant or plant virus promoters (such as CaMV). Promoters can be either constitutive, tissue specific, or developmentally expressed.

Δ9 259 Monomer Ribozyme Constructs (RPA 114, 115)

These are the Δ-9 desaturase 259 monomer hammerhead ribozyme clones. The ribozymes were designed with 3 bp long stem II and 20 bp (total) long substrate binding arms targeted against site 259. The active version is RPA 114, the inactive is RPA 115. The parent plasmid, pDAB367, was digested with Not I and filled in with Klenow to make a blunt acceptor site. The vector was then digested with *Hind* III restriction enzyme. The ribozyme containing plasmids were cut with *Eco* RI, filled-in with Klenow and recut with *Hind* III. The insert containing the entire ribozyme transcription unit was

gel-purified and ligated into the pDAB 367 vector. The constructs are checked by digestion with *Sgf* I/*Hind* III and *Xba* I/*Sst* I and confirmed by sequencing.

Δ9 453 Multimer Ribozyme Constructs (RPA 118, 119)

These are the Δ-9 desaturase 453 Multimer hammerhead ribozyme clones (see Figure 17). The ribozymes were designed with 3 bp long stem II regions. Total length of the substrate binding arms of the multimer construct was 42 bp. The active version is RPA 118, the inactive is 119. The constructs were made as described above for the 259 monomer. The multimer construct was designed with four hammerhead ribozymes targeted against sites 453, 464, 475 and 484 within Δ-9 desaturase RNA.

Δ9 252 Multimer Ribozyme Constructs (RPA 85, 113)

These are the Δ-9 desaturase 252 multimer ribozyme clones placed at the 3'end of bar (phosphoinothricin acetyl transferase; Thompson et al., 1987 *EMBO J.* 6: 2519-2523) open reading frame. The multimer contructs were designed with 3 bp long stem II regions. Total length of the substrate binding arms of the multimer construct was 91 bp. RPA 85 is the active ribozyme, RPA 113 is the inactive. The vector was constructed as follows: The parent plasmid pDAB 367 was partially digested with *Bgl* II and the single cut plasmid was gel-purified. This was recut with *Eco* RI and again gel-purified to isolate the correct *Bgl* II/*Eco* RI cut fragment. The *Bam* HI/ *Eco* RI inserts from the ribozyme constructs were gel-isolated (this contains the ribozyme and the NOS poly A region) and ligated into the 367 vector. The identitiy of positive plasmids were confirmed by performing a *Nco* I / *Sst* I digest and sequencing.

Useful transgenic plants can be identified by standard assays. The transgenic plants can be evaluated for reduction in Δ-9 desaturase expression and Δ-9 desaturase activity as discussed in the examples *infra*. .

Example 9: Identification of Potential Ribozyme Cleavage Sites in GBSS RNA

Two hundred and forty one hammer-head ribozyme sites were identified in the corn GBSS mRNA polypeptide coding region (see table IIIA). A hammer-head site consists of a uridine and any nucleotide except guanine (UH). Following is the sequence of GBSS coding region for corn (SEQ. I.D. No. 25). The numbering system starts with 1 at the 5' end of a GBSS cDNA clone having the following sequence (5' to 3'):

1

72

GACCGATCGATCGCCACAGCCAACACCACCCGCCGAGGCGACGCGACAGCCGCCA
GGAGGAAGGAATAAACT
73
144
CACTGCCAGCCAGTGAAGGGGGAGAAGTGTACTGCTCCGTCCACCAGTGCGCGCA
CCGCCCGGCAGGGCTGC
145
216
TCATCTCGTCGACGACCAGTGGATTAATCGGCATGGCGGCTCTAGCCACGTCGCA
GCTCGTCGCAACGCGCG
217
288
CCGGCCTGGGCGTCCCGGACGCGTCCACGTTCCGCCGCGGCGCCGCGCAGGGCCT
GAGGGGGGGCCGGACGG
289
360
CGTCGGCGGCGGACACGCTCAGCATTCGGACCAGCGCGCGCGCGGCGCCCAGGCT
CCAGCACCAGCAGCAGC
361
432
AGCAGGCGCGCCGCGGGGCCAGGTTCCCGTCGCTCGTCGTGTGCGCCAGCGCCGG
CATGAACGTCGTCTTCG
433
504
TCGGCGCCGAGATGGCGCCGTGGAGCAAGACCGGCGGCCTCGGCGACGTCCTCGG
CGGCCTGCCGCCGGCCA
505
576
TGGCCGCGAATGGGCACCGTGTCATGGTCGTCTCTCCCCGCTACGACCAGTACAA
GGACGCCTGGGACACCA
577
648
GCGTCGTGTCCGAGATCAAGATGGGAGACAGGTACGAGACGGTCAGGTTCTTCCA
CTGCTACAAGCGCGGAG
649
720
TGGACCGCGTGTTCGTTGACCACCCACTGTTCCTGGAGAGGGTTTGGGGAAAGAC
CGAGGAGAAGATCTACG
721
792
GGCCTGACGCTGGAACGGACTACAGGGACAACCAGCTGCGGTTCAGCCTGCTATG
CCAGGCAGCACTTGAAG
793
864
CTCCAAGGATCCTGAGCCTCAACAACAACCCATACTTCTCCGGACCATACGGGGA
GGACGTCGTGTTCGTCT
865
936

GCAACGACTGGCACACCGGCCCTCTCTCGTGCTACCTCAAGAGCAACTACCAGTCC
CACGGCATCTACAGGG

937 1008
ACGCAAAGACCGCTTTCTGCATCCACAACATCTCCTACCAGGGCCGGTTCGCCTTC
TCCGACTACCCGGAGC

1009 1080
TGAACCTCCCGGAGAGATTCAAGTCGTCCTTCGATTTCATCGACGGCTACGAGAA
GCCCGTGGAAGGCCGGA

1081 1152
AGATCAACTGGATGAAGGCCGGGATCCTCGAGGCCGACAGGGTCCTCACCGTCAG
CCCCTACTACGCCGAGG

1153 1224
AGCTCATCTCCGGCATCGCCAGGGGCTGCGAGCTCGACAACATCATGCGCCTCAC
CGGCATCACCGGCATCG

1225 1296
TCAACGGCATGGACGTCAGCGAGTGGGACCCCAGCAGGGACAAGTACATCGCCGT
GAAGTACGACGTGTCGA

1297 1368
CGGCCGTGGAGGCCAAGGCGCTGAACAAGGAGGCGCTGCAGGCGGAGGTCGGGC
TCCCGGTGGACCGGAACA

1369 1440
TCCCGCTGGTGGCGTTCATCGGCAGGCTGGAAGAGCAGAAGGGACCCGACGTCAT
GGCGGCCGCCATCCCGC

1441 1512
AGCTCATGGAGATGGTGGAGGACGTGCAGATCGTTCTGCTGGGCACGGGCAAGA
AGAAGTTCGAGCGCATGC

1513 1584
TCATGAGCGCCGAGGAGAAGTTCCCAGGCAAGGTGCGCGCCGTGGTCAAGTTCAA
CGCGGCGCTGGCGCACC

1585 1656
ACATCATGGCCGGCGCCGACGTGCTCGCCGTCACCAGCCGCTTCGAGCCCTGCGGC
CTCATCCAGCTGCAGG

1657 1728
GGATGCGATACGGAACGCCCTGCGCCTGCGCGTCCACCGGTGGACTCGTCGACAC
CATCATCGAAGGCAAGA

1729 1800

CCGGGTTCCACATGGGCCGCCTCAGCGTCGACTGCAACGTCGTGGAGCCGGCGGA
CGTCAAGAAGGTGGCCA
1801 1872
CCACCTTGCAGCGCGCCATCAAGGTGGTCGGCACGCCGGCGTACGAGGAGATGGT
GAGGAACTGCATGATCC
1873 1944
AGGATCTCTCCTGGAAGGGCCCTGCCAAGAACTGGGAGAACGTGCTGCTCAGCCT
CGGGGTCGCCGGCGGCG
1945 2016
AGCCAGGGGTCGAAGGCGAGGAGATCGCGCCGCTCGCCAAGGAGAACGTGGCCG
CGCCCTGAAGAGTTCGGC
2017 2088
CTGCAGGCCCCCTGATCTCGCGCGTGGTGCAAACATGTTGGGACATCTTCTTATAT
ATGCTGTTTCGTTTAT
2089 2160
GTGATATGGACAAGTATGTGTAGCTGCTTGCTTGTGCTAGTGTAATATAGTGTAG
TGGTGGCCAGTGGCACA
2161 2232
ACCTAATAAGCGCATGAACTAATTGCTTGCGTGTGTAGTTAAGTACCGATCGGTA
ATTTTATATTGCGAGTA
2233
AATAAATGGACCTGTAGTGGTGGAAAAAAAAAAAA (SEQ I.D. NO. 25).

There are approximately 53 potential hairpin ribozyme sites in the GBSS mRNA. The ribozyme and target sequences are listed in Table V.

Ribozymes can be readily designed and synthesized to such sites with between 5 and 100 or more bases as substrate binding arms (see Figs. 1 - 5) as described above.

Example 10: Selection of Ribozyme Cleavage Sites for GBSS

The secondary structure of GBSS mRNA was assessed by computer analysis using folding algorithms, such as the ones developed by M. Zuker (Zuker, M., 1989 *Science*, 244, 48-52. Regions of the mRNA that did not form secondary folding structures with RNA/RNA stems of over eight nucleotides and contained potential hammerhead ribozyme cleavage sites were identified.

These sites which were then assessed for oligonucleotide accessibility with RNasc H assays (see Fig. 6). Fifty-eight DNA oligonucleotides, each twenty one nucleotides long were used in these assays. These oligonucleotides covered 85 sites. The position and designation of these oligonucleotides were 195, 205, 240, 307, 390, 424, 472, 481, 539, 592, 625, 636, 678, 725, 741, 811, 859, 891, 897, 912, 918, 928, 951, 958, 969, 993, 999, 1015, 1027, 1032, 1056, 1084, 1105, 1156, 1168, 1186, 1195, 1204, 1213, 1222, 1240, 1269, 1284, 1293, 1345, 1351, 1420, 1471, 1533, 1563, 1714, 1750, 1786, 1806, 1819, 1921, 1954, and 1978. Secondary sites were also covered and included 202, 394, 384, 385, 484, 624, 627, 628, 679, 862, 901, 930, 950, 952, 967, 990, 991, 1026, 1035, 1108, 1159, 1225,1273, 1534, 1564, 1558, and 1717.

Example 11: RNaseH Assavs for GBSS

RNase H assays (Fig. 7) were performed using a full length transcript of the GBSS coding region, 3' noncoding region, and part of the 5' noncoding region. The GBSS RNA was screened for accessible cleavage sites by the method described generally in Draper *et al., supra.* hereby incorporated by reference herein. Briefly, DNA oligonucleotides representing hammerhead ribozyme cleavage sites were synthesized. A polymerase chain reaction was used to generate a substrate for T7 RNA polymerase transcription from corn cDNA clones. Labeled RNA transcripts were synthesized *in vitro* from these templates. The oligonucleotides and the labeled transcripts were annealed, RNAseH was added and the mixtures were incubated for 10 minutes at 37°C. Reactions were stopped and RNA separated on sequencing polyacrylamide gels. The percentage of the substrate cleaved was determined by autoradiographic quantitation using a phosphor imaging system (Fig. 7).

Example 12: Hammerhead Ribozymes for GBSS

Hammerhead ribozymes with 10/10 (*i.e.,* able to form 10 base pairs on each arm of the ribozyme) nucleotide binding arms were designed to the sites covered by the oligos which cleaved best in the RNase H assays. These ribozymes were then subjected to analysis by computer folding and the ribozymes that had significant secondary structure were rejected. As a result of this screening procedure 23 ribozymes were designed to the most open regions in the GBSS mRNA, the sequences of these ribozymes are shown in Table IV.

The ribozymes were chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described above (and in Usman *et al.*,

supra. Scaringe *et al.*, and Wincott *et al.*, *supra*) and are incorporated by reference herein, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were >98%. Inactive ribozymes were synthesized by substituting a U for G_5 and a U for A_{14} (numbering from (Hertel *et al.*, *supra*). Hairpin ribozymes were synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrira and Burke, 1992, *Nucleic Acids Res.*, 20, 2835-). All ribozymes were modified to enhance stability by modification of five ribonucleotides at both the 5' and 3' ends with 2'-*O*-methyl groups. Ribozymes were purified by gel electrophoresis using general methods. (Ausubel *et al.*, 1990 *Current Protocols in Molecular Biology* Wiley & Sons, NY) or were purified by high pressure liquid chromatography, as described above and were resuspended in water.

Example 13: Long Substrate Tests for GBSS

Target RNA used in this study was 900 nt long and contained cleavage sites for all the 23 HH ribozymes targeted against GBSS RNA. A template containing T7 RNA polymerase promoter upstream of GBSS target sequence, was PCR amplified from a cDNA clone. Target RNA was transcribed from this PCR amplified template using T7 RNA polymerase. The transcript was internally labeled during transcription by including [α-^{32}P] CTP as one of the four ribonucleotide triphosphates. The transcription mixture was treated with DNase-1, following transcription at 37°C for 2 hours, to digest away the DNA template used in the transcription. The transcription mixture was resolved on a denaturing polyacrylamide gel. Bands corresponding to full-length RNA was isolated from a gel slice and the RNA was precipitated with isopropanol and the pellet was stored at 4°C.

Ribozyme cleavage reactions were carried out under ribozyme excess (k_{cat}/K_M) conditions (Herschlag and Cech, *supra*). Briefly, 1000 nM ribozyme and < 10 nM internally labeled target RNA were denatured separately by heating to 90°C for 2 min. in the presence of 50 mM Tris.HCl, pH 7.5 and 10 mM $MgCl_2$. The RNAs were renatured by cooling to the reaction temperature (37°C, 26°C and 20°C) for 10-20 min. Cleavage reaction was initiated by mixing the ribozyme and target RNA at appropriate reaction temperatures. Alquots were taken at regular intervals of time and the reaction was quenched by adding equal volume of stop buffer. The samples were resolved on 4% sequencing gel.

The results from ribozyme cleavage reactions, at the three different temperatures, are summarized in Figure 8. Seven lead ribozymes were chosen (425, 892, 919, 959, 968, 1241, and 1787). One of the active ribozymes (811) produced a strange pattern of cleavage products; as a result, it was not chosen as one of our lead ribozymes.

Example 14: Cleavage of the GBSS RNA Using Multiple Ribozyme Combinations

Four of the lead ribozymes (892, 919, 959, 1241) were incubated with internally labeled target RNA in the following combinations: 892 alone; 892 + 919; 892 + 919 + 959; 892 + 919 + 959 + 1241. The fraction of RNA cleavage increased in an additive manner with an increase in the number of ribozymes used in the cleavage reaction (Fig. 9). Ribozyme cleavage reactions were carried out at 20°C as described above. These data suggest that multiple ribozymes targeted to different sites on the same mRNA will increase the reduction of target RNA in an additive manner.

Example 15: Construction of Ribozyme Expressing Transcription Units for GBSS

Cloning of GBSS Multimer Ribozymes RPA 63 (active) and RPA64 (inactive)

A multimer ribozyme was constructed which contained four hammerhead ribozymes targeting sites 892, 919, 959 and 968 of the GBSS mRNA. Two DNA oligonucleotides (Macromolecular Resourses, Fort Collins, CO) were ordered which overlap by 18 nucleotides. The sequences were as follows:

Oligo 1: CGC GGA TCC TGG TAG GAC TGA TGA GGC CGA AAG GCC GAA ATG TTG TGC TGA TGA GGC CGA AAG GCC GAA ATG CAG AAA GCG GTC TTT GCG TCC CTG TAG ATG CCG TGG C

Oligo 2: CGC GAG CTC GGC CCT CTC TTT CGG CCT TTC GGC CTC ATC AGG TGC TAC CTC AAG AGC AAC TAC CAG TTT CGG CCT TTC GGC CTC ATC AGC CAC GGC ATC TAC AGG G

Inactive versions of the above were made by substituting T for G5 and T for A14 within the catalytic core of each ribozyme motif.

These were annealed in 1 X Klenow Buffer (Gibco/BRL) at 90°C for 5 minutes and slow cooled to room temperature (22°C). NTPs were added to 0.2 mM and the oligos

extended with Klenow enzyme at 1unit/ul for one hour at 37°C. This was phenol/chloroform extracted and ethanol precipitated, then resuspended in 1X React 3 buffer (Gibco/BRL) and digested with *Bam* HI and *Sst* I for 1 hour at 37°C. The DNA was gel purified on a 2% agarose gel using the Qiagen gel extraction kit.

The DNA fragments were ligated into *Bam*HI/*Sst* I digested pDAB 353. The ligation was transformed into competent DH5α F' bacteria (Gibco/BRL). Potential clones were screened by digestion with *Bam* HI/*Eco* RI. Clones were confirmed by sequencing. The total length of homology with the target sequence is 96 nucleotides.

919 Monomer Ribozyme (RPA66)

A single ribozyme to site 919 of the GBSS mRNA was constructed with 10/10 arms as follows. Two DNA oligos were ordered:

Oligo 1: GAT CCG ATG CCG TGG CTG ATG AGG CCG AAA GGC CGA AAC TGG TAG TT

Oligo 2: AAC TAC CAG TTT CGG CCT TTC GGC CTC ATC AGC CAC GGC ATC G

The oligos are phosphorylated individually in 1X kinase buffer (Gibco/BRL) and heat denatured and annealed by combining at 90°C for 10 min, then slow cooled to room temperature (22°C). The vector was prepared by digestion of pDAB 353 with Sst I and blunting the ends with T4 DNA polymerase. The vector was redigested with *Bam* HI and gel purified as above. The annealed oligos are ligated to the vector in 1X ligation buffer (Gibco/BRL) at 16°C overnight. Potential clones were digested with *Bam* HI/*Eco* RI and confirmed by sequencing.

Example 16: Plant Transformation Plasmids pDAB 367, Used in the Δ9 Ribozyme Experiments, and pDAB353 used in the GBSS Ribozyme Experiments

Part A pDAB367

Plasmid pDAB367 has the following DNA structure: beginning with the base after the final C residue of the Sph I site of pUC 19 (base 441; Ref. 1), and reading on the strand contiguous to the LacZ gene coding strand, the linker sequence CTGCAGGCCGGCC

TTAATTAAGCGGCCGCGTTTAAACGCCCGGGCATTTAAATGGCGCGCCGC GATCGCTTGCAGATCTGCATGGGTG, nucleotides 7093 to 7344 of CaMV DNA (2), the linker sequence CATCGATG, nucleotides 7093 to 7439 of CaMV, the linker sequence GGGGACTCTAGAGGATCCAG, nucleotides 167 to 186 of MSV (3), nucleotides 188 to 277 of MSV (3), a C residue followed by nucleotides 119 to 209 of maize Adh 1S containing parts of exon 1 and intron 1 (4), nucleotides 555 to 672 containing parts of Adh 1S intron 1 and exon 2 (4), the linker sequence GACGGATCTG, and nucleotides 278 to 317 of MSV. This is followed by a modified BAR coding region from pIJ4104 (5) having the AGC serine codon in the second position replaced by a GCC alanine codon, and nucleotide 546 of the coding region changed from G to A to eliminate a Bgl II site. Next, the linker sequence TGAGATCTGAGCTCGAATTTCCCC, nucleotides 1298 to 1554 of Nos (6), and a G residue followed by the rest of the pUC 19 sequence (including the Eco RI site).

Part B pDAB353

Plasmid pDAB353 has the following DNA structure: beginning with the base after the final C residue of the Sph I site of pUC 19 (base 441; Ref. 1), and reading on the strand contiguous to the LacZ gene coding strand, the linker sequence CTGCAGATCTGCATGGGTG, nucleotides 7093 to 7344 of CaMV DNA (2), the linker sequence CATCGATG, nucleotides 7093 to 7439 of CaMV, the linker sequence GGGGACTCTAGAG, nucleotides 119 to 209 of maize Adh 1S containing parts of ex n 1 and intron 1 (4), nucleotides 555 to 672 containing parts of Adh 1S intron 1 and exon 2 (4), and the linker sequence GACGGATCCGTCGACC, where GGATCC represents the recognition sequence for BamH I restriction enzyme. This is followed by the beta-glucuronidase (GUS) gene from pRAJ275 (7), cloned as an Nco I/Sac I fragment, the linker sequence GAATTTCCCC, the poly A region in nucleotides 1298 to 1554 of Nos (6), and a G residue followed by the rest of the pUC 19 sequence (including the Eco RI site).

The following are herein incorporated by reference:

1. Messing, J. (1983) in "Methods in Enzymology" (Wu, R. *et al.*, Eds) 101:20-78.
2. Franck, A., H. Guilley, G. Jonard, K. Richards, and L. Hirth (1980) Nucleotide sequence of Cauliflower Mosaic Virus DNA. Cell 21:285-294.

3. Mullineaux, P. M., J. Donson, B. A. M. Morris-Krsinich, M. I. Boulton, and J. W. Davies (1984) The nucleotide sequence of Maize Streak Virus DNA. EMBO J. 3:3063-3068.

4. Dennis, E. S., W. L. Gerlach, A. J. Pryor, J. L. Bennetzen, A. Inglis, D. Llewellyn, M. M. Sachs, R. J. Ferl, and W. J. Peacock (1984) Molecular analysis of the alcohol dehydrogenase (*Adh1*) gene of maize. Nucl. Acids Res. 12:3983-4000.

5. White, J., S-Y Chang, M. J. Bibb, and M. J. Bibb (1990) A cassette containing the *bar* gene of *Streptomyces hygroscopicus*: a selectable marker for plant transformation. Nucl. Acids. Res. 18:1062.

6. DePicker, A., S. Stachel, P. Dhaese, P. Zambryski, and H. M. Goodman (1982) Nopaline Synthase: Transcript mapping and DNA sequence. J. Molec. Appl. Genet. 1:561-573.

7. Jefferson, R. A. (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molec. Biol. Reporter 5:387-405.

Example 17: Plasmid pDAB359 a Plant Transformation Plasmid which Contains the Gamma-Zein Promoter, the Antisense GBSS, and a the Nos Polyadenylation Sequence

Plasmid pDAB359 is a 6702 bp double-stranded, circular DNA that contains the following sequence elements: nucleotides 1-404 from pUC18 which include lac operon sequence from base 238 to base 404 and ends with the HindIII site of the M13mp18 polylinker (1,2); the Nos polyadenylation sequence from nucleotides 412 to 668 (3); a synthetic adapter sequence from nucleotides 679-690 which converts a Sac I site to an Xho I site by changing GAGCTC to GAGCTT and adding CTCGAG; maize granule bound starch synthase cDNA from bases 691 to 2953, corresponding to nucleotides 1-2255 of SEQ. I.D. No. 25. The GBSS sequence in plasmid pDAB359 was modified from the original cDNA by the addition of a 5' Xho I and a 3' Nco I site as well as the deletion of internal Nco I and Xho I sites using Klenow to fill in the enzyme recognition sequences. Bases 2971 to 4453 are 5' untranslated sequence of the maize 27 kD gamma-zein gene corresponding to nucleotides 1078 to 2565 of the published sequence (4). The gamma-zein sequence was modified to contain a 5' Kpn I site and 3' BamH/SalI/Nco I sites. Additional changes in the gamma-zein sequence relative to the published sequence include a T deletion at nucleotide 104, a TACA deletion at nucleotide 613, a C to T conversion at nucleotide 812, an A deletion at nucleotide 1165 and an A insertion at nucleotide 1353. Finally, nucleotides 4454 to 6720 of pDAB359 are identical to puc18 bases 456 to 2686 including the Kpn I/EcoRI/Sac I sites of the M13/mp18 polylinker

from 4454 to 4471, a lac operon fragment from 4471 to 4697, and the ß-lacatmase gene from 5642 to 6433 (1, 2).

The following are incorporated by reference herein:

pUC18- Norrander, J., Kempe, T., Messing, J. Gene (1983) 26: 101-106; Pouwels, P.H., Enger-Valk, B.E., Brammar, W. J. Cloning Vectors, Elsevier 1985 and supplements

NosA - DePicker, A., Stachel, S., Dhaese, P., Zambryski, P., and Goodman, H.M. (1982) Nopaline Synthase: Transcript Mapping and DNA Sequence J. Molec. Appl. Genet. 1:561-573.

Maize 27kD gamma-zein - Das, O.P., Poliak, E.L., Ward, K., Messing, J. Nucleic Acids Research 19, 3325 - 3330 (1991).

Example 18: Construction of Plasmid pDAB430, containing Antisense Δ9 Desaturase, Expressed by the Ubiquitin Promoter/intron (Ubi1)

Part A Construction of plasmid pDAB421

Plasmid pDAB421 contains a unique blunt-end *SrfI* cloning site flanked by the maize Ubiquitin promoter/intron and the nopaline synthase polyadenylation sequences. pDAB421 was prepared as follows: digestion of pDAB355 with restriction enzymes *KpnI* and *BamHI* drops out the R coding region on a 2.2 kB fragment. Following gel purification, the vector was ligated to an adapter composed of two annealed oligonucleotides OF235 and OF236. OF235 has the sequence 5' - GAT CCG CCC GGG GCC CGG GCG GTA C - 3' and OF236 has the sequence 5' - CGC CCG GGC CCC GGG CG - 3'. Clones containing this adapter were identified by digestion and linearization of plasmid DNA with the enzymes *SrfI* and *SmaI* which cut in the adapter, but not elsewhere in the plasmid. One representative clone was sequenced to verify that only one adapter was inserted into the plasmid. The resulting plasmid pDAB421 was used in subsequent construction of the Δ9 desaturase antisense plasmid pDAB430.

Part B Construction of plasmid pDAB430 (antisense Δ9 desaturase)

The antisense Δ9 desaturase construct present in plasmid pDAB430 was produced by cloning of an amplification product in the blunt-end cloning site of plasmid pDAB421. Two constructs were produced simultaneously from the same experiment. The first

construct contains the Δ9 desaturase gene in the sense orientation behind the ubiquitin promoter, and the c-myc tag fused to the C-terminus of the Δ9 desaturase open reading frame for immunological detection of overproduced protein in transgenic lines. This construct was intended for testing of ribozymes in a system which did not express maize Δ9 desaturase. This construct was never used, but the primers used to amplify and construct the Δ9 desaturase antisense gene were the same. The Δ9 desaturase cDNA sequence described herein was amplified with two primers. The N-terminal primer OF279 has the sequence 5'- GTG CCC ACA ATG GCG CTC CGC CTC AAC GAC - 3'. The underlined bases correspond to nucleotides 146-166 of the cDNA sequence. C-terminal primer OF280 has the sequence 5' - TCA TCA CAG GTC CTC CTC GCT GAT CAG CTT CTC CTC CAG TTG GAC CTG CCT ACC GTA - 3' and is the reverse complement of the sequence 5' - TAC GGT AGG GAC GTC CAA CTG *GAG GAG AAG CTG ATC AGC GAG GAG GAC CTG* TGA TGA - 3'. In this sequence the underlined bases correspond to nucleotides 1304-1324 of the cDNA sequence, the bases in italics correspond to the sequence of the c-myc epitope. The 1179 bp of amplification product was purified through a 1.0% agarose gel, and ligated into plasmid pDAB421 which was linearized with the restriction enzyme *Srf I*. Colony hybridization was used to select clones containing the pDAB421 plasmid with the insert. The orientation of the insert was determined by restriction digestion of plasmid DNA with diagnostic enzymes *KpnI* and *BamHI*. A clone containing the Δ9 desaturase coding sequence in the sense orientation relative to the Ubiquitin promoter/intron was recovered and was named pDAB429. An additional clone containing the Δ9 desaturase coding sequence in the anitsense orientation relative to the promoter was named pDAB430. Plasmid pDAB430 was subjected to sequence analysis and it was determined that the sequence contained three PCR induced errors compared to the expected sequence. One error was found in the sequence corresponding to primer OF280 and two nucleotide changes were observed internal to the coding sequence. These errors were not corrected, because antisense downregulation does not require 100% sequence identity between the antisense transcript and the downregulation target.

Example 19: Helium Blasting of Embryogenic Maize Cultures and the Subsequent Regeneration of Transgenic Progeny

Part A Establishment of embryogenic maize cultures. The tissue cultures employed in transformation experiments were initiated from immature zygotic embryos of the genotype "Hi-II". Hi-II is a hybrid made by intermating 2 R_3 lines derived from a

B73xA188 cross (Armstrong et al. 1990). When cultured, this genotype produces callus tissue known as Type II. Type II callus is friable, grows quickly, and exhibits the ability to maintain a high level of embryogenic activity over an extended time period.

Type II cultures were initiated from 1.5-3.0 mm immature embryos resulting from controlled pollinations of greenhouse grown Hi-II plants. The initiation medium used was N6 (Chu, 1978) which contained 1.0mg/L 2,4-D, 25 mM L-proline, 100 mg/L casein hydrolysate, 10 mg/L $AgNO_3$, 2.5 g/L gelrite and 2% sucrose adjusted to pH 5.8. For approximately 2-8 weeks, selection occurred for Type II callus and against non-embryogenic and/or Type I callus. Once Type II callus was selected, it was transferred to a maintenance medium in which $AgNO_3$ was omitted and L-proline reduced to 6mM.

After approximately 3 months of subculture in which the quantity and quality of embryogenic cultures was increased, the cultures were deemed acceptable for use in transformation experiments.

Part B Preparation of plasmid DNA. Plasmid DNA was adsorbed onto the surface of gold particles prior to use in transformation experiments. The experiments for the GBSS target used gold particles which were spherical with diameters ranging from 1.5-3.0 microns (Aldrich Chemical Co., Milwaukee, WI). Transfomation experiments for the Δ9 desaturase target used 1.0 micron spherical gold particles (Bio-Rad, Hercules, CA). All gold particles were surface-sterilized with ethanol prior to use. Adsorption was accomplished by adding 74 µl of 2.5 M calcium chloride and 30 µl of 0.1 M spermidine to 300 µl of plasmid DNA and sterile H_2O. The concentration of plasmid DNA was 140 µg. The DNA-coated gold particles were immediately vortexed and allowed to settle out of suspension. The resulting clear supernatent was removed and the particles were resuspended in 1 ml of 100% ethanol. The final dilution of the suspension ready for use in helium blasting was 7.5 mg DNA/gold per ml of ethanol.

Part C Preparation and helium blasting of tissue targets. Approximately 600 mg of embryogenic callus tissue per target was spread over the surface of petri plates containing Type II callus maintenance medium plus 0.2 M sorbitol and 0.2 M mannitol as an osmoticum. After an approximately 4 hour pretreatment, all tissue was transferred to petri plates containing 2% agar blasting medium (maintenance medium plus osmoticum plus 2% agar).

Helium blasting involved accelerating the suspended DNA-coated gold particles towards and into prepared tissue targets. The device used was an earlier prototype to the one described in a DowElanco U.S. Patent (#5,141,131) which is incorporated herein by reference, although both function in a similar manner. The device consisted of a high pressure helium source, a syringe containing the DNA/gold suspension, and a pneumatically-operated multipurpose valve which provided controlled linkage between the helium source and a loop of pre-loaded DNA/gold suspension.

Prior to blasting, tissue targets were covered with a sterile 104 micron stainless steel screen, which held the tissue in place during impact. Next, targets were placed under vacuum in the main chamber of the device. The DNA-coated gold particles were accelerated at the target 4 times using a helium pressure of 1500 psi. Each blast delivered 20 µl of DNA/gold suspension. Immediately post-blasting, the targets were placed back on maintenance medium plus osmoticum for a 16 to 24 hour recovery period.

Part D Selection of transformed tissue and the regeneration of plants from transgenic cultures. After 16 to 24 hours post-blasting, the tissue was divided into small pieces and transferred to selection medium (maintenance medium plus 30 mg/L Basta™). Every 4 weeks for 3 months, the tissue pieces were non-selectively transferred to fresh selection medium. After 8 weeks and up to 24 weeks, any sectors found proliferating against a background of growth inhibited tissue were removed and isolated. Putatively transformed tissue was subcultured onto fresh selection medium. Transgenic cultures were established after 1 to 3 additional subcultures.

Once Basta™ resistant callus was established as a line, plant regeneration was initiated by transferring callus tissue to petri plate containing cytokinin-based induction medium which were then placed in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). The induction medium was composed of MS salts and vitamins (Murashige and Skoog, 1962), 30 g/L sucrose, 100 mg/L myo-inositol, 5 mg/L 6-benzylaminopurine, 0.025 mg/L 2,4-D, 2.5 g/L gelrite adjusted to pH 5.7. Following the two week induction period, the tissue was non-selectively transferred to hormone-free regeneration medium and kept in high light. The regeneration medium was composed of MS salts and vitamins, 30 g/L sucrose and 2.5 g/L gelrite adjusted to pH 5.7. Both induction and regeneration media contained 30 mg/L Basta™. Tissue began differentiating shoots and roots in 2-4 weeks. Small (1.5-3 cm) plantlets were removed and placed in tubes containing SH medium. SH medium is composed of SH salts and vitamins (Schenk

and Hildebrandt, 1972). 10 g/L sucrose, 100 mg/L myo-inositol, 5 mL/L FeEDTA, and either 7 g/L Agar or 2.5 g/L Gelrite adjusted to pH 5.8. Plantlets were transferred to 10 cm pots containing approximately 0.1 kg of Metro-Mix® 360 (The Scotts Co., Marysville, OH) in the greenhouse as soon as they exhibited growth and developed a sufficient root system (1-2 weeks). At the 3-5 leaf stage, plants were transferred to 5 gallon pots containing approximately 4 kg Metro-Mix® 360 and grown to maturity. These R_0 plants were self-pollinated and/or cross-pollinated with non-transgenic inbreds to obtain transgenic progeny. In the case of transgenic plants produced for the GBSS target, R_1 seed produced from R_0 pollinations was replanted. The R_1 plants were grown to maturity and pollinated to produce R_2 seed in the quantities needed for the analyses.

Example 20: Production and Regeneration of Δ9 Transgenic Material.

Part A Transformation and isolation of embryogenic callus. Six ribozyme constructs, described previously, targeted to Δ9 desaturase were transformed into regenerable Type II callus cultures as described herein. These 6 constructs consisted of 3 active/inactive pairs; namely, RPA85/RPA113, RPA114/RPA115, and RPA118/RPA119. A total of 1621 tissue targets were prepared, blasted, and placed into selection. From these blasting experiments 334 independent Basta®-resistant transformation events ("lines") were isolated from selection. Approximately 50% of these lines were analyzed via DNA PCR or GC/FAME as a means of determining which ones to move forward to regeneration and which ones to discard. The remaining 50% were not analyzed either because they had become non-embryogenic or contaminated.

Part B Regeneration of Δ9 plants from transgenic callus. Following analyses of the transgenic callus, twelve lines were chosen per ribozyme construct for regeneration, with 15 R_0 plants to be produced per line. These lines generally consisted of 10 analysis-positive lines plus 2 negative controls, however, due to the poor regenerability of some of the cultures, plants were produced from less than 12 lines for constructs RPA113, RPA115, RPA118, and RPA119. An overall total of 854 R_0 plants were regenerated from 66 individual lines (see Table X). When the plants reached maturity, self- or sib-pollinations were given the highest priority, however, when this was not possible, cross-pollinations were made using the inbreds CQ806, CS716, OQ414, or HO_1 as pollen donors, and occasionally as pollen recipients. Over 715 controlled pollinations have been made, with the majority (55%) being comprised of self- or sib-pollinations and the

minority (45%) being comprised of F1 crosses. R_1 seed was collected approximately 45 days post-pollination.

Example 21: Production and Regeneration of Transgenic Maize for the GBSS

Part A Transformation of embryogenic maize callus and the subsequent selection and establishment of transgenic cultures. RPA63 and RPA64, an active/inactive pair of ribozyme multimers targeted to GBSS, were inserted along with *bar* selection plasmid pDAB308 into Type II callus as described herein. A total of 115 Basta™-resistant independent transformation events were recovered from the selection of 590 blasted tissue targets. Southern analysis was performed on callus samples from established cultures of all events to determine the status of the gene of interest.

Part B Regeneration of plants from cultures transformed with ribozymes targeted to GBSS as well as the advancement to the R_2 generation. Plants were regenerated from Southern "positive" transgenic cultures and grown to maturity in a greenhouse. The primary regenerates were pollinated to produce R_1 seed. From 30 to 45 days after pollination, seed was harvested, dried to the correct moisture content, and replanted. A total of 752 R_1 plants, representing 16 original lines, were grown to sexual maturity and pollinated. Approximately 19 to 22 days after pollination, ears were harvested and 30 kernels were randomly excised per ear and frozen for later analyses.

Example 22: Testing of GBSS-Targeted Ribozymes in Maize Black Mexican Sweet (BMS) Stably Transformed Callus

Part A Production of BMS callus stably transformed with GBSS and GBSS-targeted ribozymes. BMS does not produce a GBSS mRNA which is homologous to that found endogenously in maize. Therefore, a double transformation system was developed to produce transformants which expressed both target and ribozymes. "ZM" BMS suspensions (obtained from Jack Widholm, University of Illinois, also see W. F. Sheridan, "Black Mexican Sweet Corn: Its Use for Tissue Cultures" in *Maize for Biological Research,* W. F. Sheridan, editor. University Press. University of North Dakokta, Grand Forks, ND, 1982, pp. 385-388) were prepared for helium blasting four days after subculture by transfer to a 100 x 20 mm Petri plate (Fisher Scientific, Pittsburgh, PA) and partial removal of liquid medium, forming a thin paste of cells. Targets consisted of 100-125 mg fresh weight of cells on a 1/2" antibiotic disc (Schleicher and Schuell, Keene, NH)

placed on blasting medium. DN6 [N6 salts and vitamins (Chu *et al.*, 1978), 20 g/L sucrose, 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 25 mM L-proline; pH= 5.8 before autoclaving 20 minutes at 121°C] solidified with 2% TC agar (JRH Biosciences, Lenexa, Kansas) in 60 x 20 mm plates. DNA was precipitated onto gold particles. For the first transformation, pDAB 426 (Ubi/GBSS) and pDAB 308 (35T/Bar) were used. Targets were individually shot using DowElanco Helium Blasting Device I. With a vacuum pressure of 650 mm Hg and at a distance of 15.5 cm from target to device nozzle, each sample was blasted once with DNA/gold mixture at 500 psi. Immediately after blasting, the antibiotic discs were transferred to DN6 medium made with 0.8% TC agar for one week of target tissue recovery. After recovery, each target was spread onto a 5.5 cm Whatman #4 filter placed on DN6 medium minus proline with 3 mg/L Basta® (Hoechst, Frankfort, Germany). Two weeks later, the filters were transferred to fresh selection medium with 6 mg/L Basta®. Subsequent transfers were done at two week intervals. Isolates were picked from the filters and placed on AMCF-ARM medium (N6 salts and vitamins, 20 g/L sucrose, 30 g/L mannitol, 100 mg/L acid casein hydrolysate, and 1 mg/L 2,4-D, 24 mM L-proline; pH= 5.8 before autoclaving 20 minutes at 121°C) solidified with 0.8% TC agar containing 6 mg/L Basta®. Isolates were maintained by subculture to fresh medium every two weeks.

Basta®-resistant isolates which expressed GBSS were subjected to a second transformation. As with BMS suspensions, targets of transgenic callus were prepared 4 days after subculture by spreading tissue onto 1/2" filters. However, AMCF-ARM with 2% TC agar was used for blasting, due to maintenance of transformants on AMCF-ARM selection media. Each sample was covered with a sterile 104 µm mesh screen and blasting was done at 1500 psi. Target tissue was co-bombarded with pDAB 319 (35S-ALS; 35T-GUS) and RPA63 (active ribozyme multimer) or pDAB319 and RPA64 (inactive ribozyme multimer), or shot with pDAB 319 alone. Immediately after blasting, all targets were transferred to nonselective medium (AMCF-ARM) for one week of recovery. Subsequently, the targets were placed on AMCF-ARM medium containing two selection agents, 6 mg/L Basta® and 2 µg/L chlorsulfuron (CSN). The level of CSN was increased to 4 ug/L after 2 weeks. Continued transfer of the filters and generation of isolates was done as described in the first transformation, with isolates being maintained on AMCF-ARM medium containing 6 mg/L Basta and 4 µg/L CSN.

Part B Analysis of BMS stable transformants expressing GBSS and GBSS-targeted ribozymes. Isolates from the first transformation were evaluated by Northern blot

analysis for detection of a functional target gene (GBSS) and to determine relative levels of expression. In 12 of 25 isolates analyzed, GBSS transcript was detected. A range of expression was observed, indicating an independence of transformation events. Isolates generated from the second transformation were evaluated by Northern blot analysis for detection of continued GBSS expression and by RT-PCR to screen for the presence of ribozyme transcript. Of 19 isolates tested from one previously transformed line, 18 expressed the active ribozyme, RPA63, and all expressed GBSS. GBSS was detected in each of 6 vector controls; ribozyme was not expressed in these samples. As described herein, RNase protection assay (RPA) and Northern blot analysis were performed on ribozyme-expressing and vector control tissues to compare levels of GBSS transcript in the presence or absence of active ribozyme. GBSS values were normalized to an internal control (Δ9 desaturase); Northern blot data is shown in Figure (25). Northern blot results revealed a significantly lower level of GBSS message in the presence of ribozyme, as compared to vector controls. RPA data showed that some of the individual samples expressing active ribozyme ("L" and "O") were significantly different from vector controls and similar to a nontransformed control.

Example 23: Analysis of Plant and Callus Materials

Plant material co-transformed with the pDAB308 and one of the following ribozyme containing vectors, pRPA63, pRPA64, pRPA85, pRPA113, pRPA114, pRPA115, pRPA118 or pRPA119 were analyzed at the callus level, Ro level and select lines analyzed at the F1 level. Leaf material was harvested when the plantlets reached the 6-8 leaf stage. DNA from the plant and callus material was prepared from lyophilized tissue as described by Saghai-Maroof *et al.*(*supra*). Eight micrograms of each DNA was digested with the restriction enzymes specific for each construct using conditions suggested by the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD) and separated by agarose gel electrophoresis. The DNA was blotted onto nylon membrane as described by Southern, E. 1975 "Detection of specific sequences among DNA fragments separated by gel electrophoresis," J Mol. Biol. 98:503 and Southern, E. 1980 "Gel electrophoresis of restriction fragments" Methods Enzmol. 69:152, which are incorporated by reference herein.

Probes specific for the ribozyme coding region were hybridized to the membranes. Probe DNA was prepared by boiling 50 ng of probe DNA for 10 minutes then quick cooling on ice before being added to the Ready-To-Go DNA labeling beads (Pharmacia

LKB, Piscataway, NJ) with 50 microcuries of $\alpha^{32}P$-dCTP (Amersham Life Science, Arlington Heights, IL). Probes were hybridized to the genomic DNA on the nylon membranes. The membranes were washed at 60°C in 0.25X SSC and 0.2% SDS for 45 minutes, blotted dry and exposed to XAR-5 film overnight with two intensifying screens.

The DNA from the RPA63 and RPA64 was digested with the restriction enzymes HindIII and EcoRI and the blots containing these samples were hybridized to the RPA63 probe. The RPA63 probe consists of the RPA63 ribozyme multimer coding region and should produce a single 1.3 kb hybridization product when hybridized to the RPA63 or RPA64 materials. The 1.3 kb hybridization product should contain the enhanced 35S promoter, the AdhI intron, the ribozyme coding region and the nopaline synthase poly A 3' end. The DNA from the RPA85 and RPA113 was digested with the restriction enzymes HindIII and EcoRI and the blots containing these samples were hybridized to the RPA122 probe. RPA 122 is the 252 multimer ribozyme in pDAB 353 replacing the GUS reporter. The RPA122 probe consists of the RPA122 ribozyme multimer coding region ~~and the nopaline synthase~~ 3' end and should produce a single 2.1 kb hybridization product when hybridized to the RPA85 or RPA113 materials. The 2.1 kb hybridization product should contain the enhanced 35S promoter, the AdhI intron, the bar gene, the ribozyme coding region and the nopaline synthase poly A 3' end. The DNA from the RPA114 and RPA115 was digested with the restriction enzymes HindIII and SmaI and the blots containing these samples were hybridized to the RPA115 probe. The RPA115 probe consist of the RPA115 ribozyme coding region and should produce a single 1.2 kb hybridization product when hybridized to the RPA114 or RPA115 materials. The 1.2 kb hybridization product should contain the enhanced 35S promoter, the AdhI intron, the ribozyme coding region and the nopaline synthase poly A 3' end. The DNA from the RPA118 and RPA119 was digested with the restriction enzymes HindIII and SmaI and the blots containing these samples were hybridized to the RPA118 probe. The RPA118 probe consist of the RPA118 ribozyme coding region and should produce a single 1.3 kb hybridization product when hybridized to the RPA118 or RPA119 materials. The 1.3 kb hybridization product should contain the enhanced 35S promoter, the AdhI intron, the ribozyme coding region and the nopaline synthase poly A 3' end.

Example 24: Extraction of Genomic DNA from Transgenic Callus

Three hundred mg of actively growing callus were quick frozen on dry ice. It was ground to a fine powder with a chilled Bessman Tissue Pulverizer (Spectrum, Houston,

TX) and extracted with 400µl of 2x CTAB buffer (2% Hexadecyltrimethylammonium Bromide, 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone). The suspension was lysed at 65°C for 25 minutes, then extracted with an equal volume of chloroform:isoamyl alcohol. To the aqueous phase was added 0.1 volumes of 10% CTAB buffer (10% Hexadecyltrimethylammonium Bromide, 0.7 M NaCl). Following extraction with an equal volume of chloroform:isoamyl alcohol, 0.6 volumes of cold isopropyl alcohol was added to the aqueous phase, and placed at -20°C for 30 minutes. After a 5 minute centrifugation at 14,000 rpm, the resulting precipitant was dried for 10 minutes under vacuum. It was resuspended in 200 µl TE (10mM Tris, 1mMEDTA, pH 8.0) at 65°C for 20 minutes. 20% Chelex (Biorad,) was added to the DNA to a final concentration of 5% and incubated at 56°C for 15-30 minutes to remove impurities. The DNA concentration was measured on a Hoefer Fluorimeter (Hoefer, San Francisco).

Example 25: PCR Analysis of Genomic Callus DNA

Use of Polymerase Chain Reaction (PCR) to demonstrate the stable insertion of ribozyme genes into the chromosome of transgenic maize calli.

Part A Method used to detect ribozyme DNA The Polymerase Chain Reaction (PCR) was performed as described in the suppliers protocol using AmpliTaq DNA Polymerase (GeneAmp PCR kit, Perkin Elmer, Cetus). Aliquots of 300 ng of genomic callus DNA, 1 µl of a 50 µM downstream primer (5' CGC AAG ACC GGC AAC AGG 3'), 1µl of an upstream primer and 1µl of Perfect Match (Stratagene, Ca) PCR enhancer were mixed with the components of the kit. The PCR reaction was performed for 40 cycles using the following parameters; denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 3 mins. An aliquot of 0.2x vol. of each PCR reaction was electrophoresised on a 2% 3:1 Agarose (FMC) gel using standard TAE agarose gel conditions.

Part B Upstream primer used for detection of Δ9 desaturase ribozyme genes

RPA85/RPA113 251 multimer fused to BAR 3' ORF
RPA114/RPA115 258 ribozyme monomer
RPA118/RPA119 452 ribozyme multimer
5' TGG ATT GAT GTG ATA TCT CCA C 3'

This primer is used to amplify across the Eco RV site in the 35S promoter.

Primers were prepared using standard oligo synthesis protocols on an Applied Biosystems Model 394 DNA/RNA synthesizer.

Example 26: Preparation of Total RNA from Transgenic Maize Calli and Plant

Part A Preparation of total RNA from transgenic non-regenerable and regenerable callus tissue. Three hundred milligrams of actively growing callus was quick frozen on dry ice. The tissue was ground to a fine powder with a chilled Bessman Tissue Pulverizer (Spectrum, Houston, TX) and extracted with RNA Extraction Buffer (50 mM Tris-HCl pH 8.0, 4% para-amino salicylic acid, 1% Tri-iso-propylnapthalenesulfonic acid, 10 mM dithiothreitol, and 10 mM Sodium meta-bisulfite) by vigorous vortexing. The homogenate was then extracted with an equal volume of phenol containing 0.1% 8-hydroxyquinoline. After centrifugation, the aqueous layer was extracted with an equal volume of phenol containing chloroform:isoamyl alcohol (24:1), followed by extraction with chloroform:octanol (24:1). Subsequently, 7.5 M Ammonium acetate was added to a final concentration of 2.5 M, the RNA was precipitated for 1 to 3 hours at 4°C. Following 4°C centrifugation at 14,000 rpm, RNA was resuspended in sterile water, precipitated with 2.5 M NH_4OAc and 2 volumes of 100% ethanol and incubated overnite at -20°C. The harvested RNA pellet was washed with 70% ethanol and dried under vacuum. RNA was resuspended in sterile H_2O and stored at -80°C.

Part B Preparation of total RNA from transgenic maize plants. A five cm section (~150 mg) of actively growing maize leaf tissue was excised and quick frozen in dry ice. The leaf was ground to a fine powder in a chilled mortar. Following manufactorers instructions, total RNA was purified from the powder using a Qaigen RNeasy Plant Total RNA kit (Qiagen Inc., Chatsworth, CA). Total RNA was released from the RNeasy columns by two sequential elution spins of prewarmed (50°C) sterile water (30 µl each) and stored at - 80°C.

Example 27: Use of RT-PCR Analysis to Demonstrate Expression of Ribozyme RNA in Transgenic Maize Calli and Plants

Part A Method used to detect ribozyme RNA. The Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed as described in the suppliers protocol using a thermostable rTth DNA Polymerase (rTth DNA Polymerase RNA PCR kit, Perkin Elmer Cetus). Aliquots of 300 ng of total RNA (leaf or callus) and 1 µl of a 15 µM

downstream primer (5' CGC AAG ACC GGC AAC AGG 3') were mixed with the RT components of the kit. The reverse transcription reaction was performed in a 3 step ramp up with 5 minute incubations at 60°C, 65°C, and 70°C. For the PCR reaction, 1μl of upstream primer specific for the ribozyme RNA being analyzed was added to the RT reaction with the PCR components. The PCR reaction was performed for 35 cycles using the following parameters; incubation at 96°C for 1 minute, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 3 mins. An aliquot of 0.2x vol. of each RT-PCR reaction was electrophoresed on a 2% 3:1 Agarose (FMC) gel using standard TAE agarose gel conditions.

Part B Specific upstream primers used for detection of GBSS ribozymes.

GBSS Active and Inactive Multimer

5' CAG ATC AAG TGC AAA GCT GCG GAC GGA TCT G 3'

This primer covers the Adh I intron footprint upstream of the first ribozyme arm.

GBSS 918 Intron (-) Monomer:

5' ATC CGA TGC CGT GGC TGA TG 3'

This primer covers the 10 base pair ribozyme arm and the first 6 bases of the ribozyme catalytic domain.

GBSS ribozyme expression in transgenic callus and plants was confirmed by RT-PCR.

GBSS multimer ribozyme expression in stably transformed callus was also determined by Ribonuclease Protection Assay.

Part C Specific upstream primers used for detection of Δ9 desaturase ribozymes.

RPA85/RPA113 252 multimer fused to BAR 3' ORF

5' GAT GAG ATC CGG TGG CAT TG 3'

This primer spans the junction of the BAR gene and the RPA85/113 ribozyme.

RPA114/RPA115 259 ribozyme monomer

5' ATC CCC TTG GTG GAC TGA TG 3'

This primer covers the 10 base pair ribozyme arm and the first 6 bases of the ribozyme catalytic domain.

RPA118/RPA119 453 ribozyme multimer

5' CAG ATC AAG TGC AAA GCT GCG GAC GGA TCT G 3'

This primer covers the Adh I intron footprint upstream of the first ribozyme arm.

Expression of Δ9 desaturase ribozymes in transgenic plant lines 85-06, 113-06 and 85-15 were confirmed by RT-PCR.

Primers were prepared using standard oligo synthesis protocols on an Applied Biosystems Model 394 DNA/RNA synthesizer.

Example 28: Demonstration of Ribozyme Mediated Reduction in Target mRNA Levels in Transgenic Maize Callus and Plants

Part A Northern analysis method which was used to demonstrated reductions in target mRNA levels. Five µg of total RNA was dried under vacuum, resuspended in loading buffer (20mM phosphate buffer pH 6.8, 5mM EDTA; 50% formamide: 16% formaldehyde: 10% glycerol) and denatured for 10 minutes at 65°C. Electrophoresis was at 50 volts through 1 % agarose gel in 20 mM phosphate buffer (pH 6.8) with buffer recirculation. BRL 0.24-9.5 Kb RNA ladder (Gibco/BRL, Gaithersburg, MD) were stained in gels with ethiduim bromide. RNA was transferred to GeneScreen membrane filter (DuPont NEN, Boston MA) by capillary transfer with sterile water. Hybridization was performed as described by DeLeon et al. (1983) at 42 °C, the filters were washed at 55 °C to remove non-hybridized probe. The blot was probed sequentially with cDNA fragments from the target gene and an internal RNA control gene. The internal RNA standard was utilized to distinguish variation in target mRNA levels due to loading or handling errors from true ribozyme mediated RNA reductions. For each sample the level of target mRNA was compared to the level of control mRNA within that sample. Fragments were purified by Qiaex resin (Qaigen Inc. Chatsworth, CA) from lx TAE agarose gels. They were nick-translated using an Amersham Nick Translation Kit (Amersham Corporation, Arlington Heights , Ill.) with alpha ^{32}P dCTP. Autoradiography was at -70° C with intensifying screens (DuPont, Wilmington DE) for one to three days. Autoradiogram signals for each probe were measured after a 24 hour exposure by densitometer and a ratio of target/internal control mRNA levels was calculated.

Ribonuclease protection assays were performed as follows: RNA was prepared using the Qiagen RNeasy Plant Total RNA Kit from either BMS protoplasts or callus material. The probes were made using the Ambion Maxiscript kit and were typically 10^8 cpm/ microgram or higher. The probes were made the same day they were used. They were gel purified, resuspended in RNase-free10mM Tris (pH 8) and kept on ice. Probes were diluted to $5x10^5$cpm/ul immediately before use. 5 µg of RNA derived from callus or 20 µg of RNA derived from protoplasts was incubated with 5 x 10^5 cpm of probe in 4M Guanidine Buffer. [4M Guanidine Buffer: 4M Guanidine Thiocyanate/0.5%

Sarcosyl/25mM Sodium Citrate (pH 7.4)]. 40 ul of PCR mineral oil was added to each tube to prevent evaporation. The samples were heated to 95° for 3 minutes and placed immediately into a 45° water bath. Incubation continued overnight. 600 µl of RNase Treatment Mix was added per sample and incubated for 30 minutes at 37°C. (RNase Treatment Mix: 400 mM NaCl, 40 units/ml RNase A and T1). 12 µl of 20% SDS were added per tube, immediately followed by addition of 12 ul (20 mg/ml) Proteinase K to each tube. The tubes were vortexed gently and incubated for 30 minutes at 37°C. 750 ul of room temperature RNase-free isopropanol was added to each tube, and mixed by inverting repeatedly to get the SDS into solution. The samples were then microfuged at top speed at room temperature for 20 minutes. The pellets were air dried for 45 minutes. 15 ul of RNA Running Buffer was added to each tube, and vortexed hard for 30 seconds. (RNA Running Buffer: 95% Formamide/20mM EDTA/0.1% Bromophenol Blue/0.1% Xylene Cyanol). The sample was heated to 95° C for 3 minutes, and loaded onto an 8% denaturing acrylamide gel. The gel was vacuum dried and exposed to a phosphorimager screens for 4 to 12 hours.

Part B Results demonstrating reductions in GBSS mRNA levels in nongenerable callus expressing both a GBSS and GBSS targeted ribozyme RNA. The production of nonregenerable callus expressing RNAs for the GBSS target gene and an active multimer ribozyme targeted to GBSS mRNA was performed. Also produced were transgenics expressing GBSS and a ribozyme (-) control RNA. Total RNA was prepared from the transgenic lines. Northern analysis was performed on 7 ribozyme (-) control transformants and 8 active RPA63 lines. Probes for this analysis were a full length maize GBSS cDNA and a maize Δ9 cDNA fragment. To distinguish variation in GBSS mRNA levels due to loading or handling errors from true ribozyme mediated RNA reductions, the level of GBSS mRNA was compared to the level of Δ9 mRNA within that sample. The level of full length GBSS transcript was compared between ribozyme expressing and ribozyme minus calli to identify lines with ribozyme mediated target RNA reductions. Blot to blot variation was controlled by performing duplicate analyses.

A range in GBSS/ Δ9 ratio was observed between ribozyme (-) transgenics. The target mRNA is produced by a transgene and may be subject to more variation in expression then the endogenous Δ9 mRNA. Active lines (RPA 63) AA, EE, KK, and JJ were shown to reduce the level of GBSS/Δ9 most significantly, as much as 10 fold as compared to ribozyme (-) control transgenics this is graphed in Figure 25. Those active

lines were shown to be expressing GBSS targeted ribozyme by RT-PCR as described herein.

Reductions in GBSS mRNA compared to Δ9 mRNA were also seen by RNAse protection assay.

Part C Demonstration of reductions in Δ9 desaturase levels in transgenic plants expressing ribozymes targeted to Δ9 desaturase mRNA. The high stearate transgenics, RPA85-06 and RPA85-15, each contained an intact copy of the fused ribozyme multimer gene. Within each line, plants were screened by RT-PCR for the presence of ribozyme RNA. Using the protocol described in Example 27. RPA85 ribozyme expression was demonstrated in plants of the 85-06 and 85-15 lines which contained high stearic acid in their leaves. Northern analysis was performed on the six high stearate plants from each line as well as non-transformed (NT) and transformed control (TC) plants. The probes for this analysis were cDNA fragments from a maize Δ9 desaturase cDNA and a maize actin cDNA. To distinguish variation in Δ9 mRNA levels due to loading or handling errors from true ribozyme mediated RNA reductions, the level of Δ9 mRNA was compared to the level of actin mRNA within that sample. Using densitometer readings described above a ratio was calculated for each sample. Δ9/actin ratio values ranging from 0.55 to 0.88 were calculated for the 85-06 plants. The average Δ9/actin value for non-transformed controls was 2.7. There is an apparent 4 fold reduction in Δ9/actin ratios between 85-06 and NT leaves. Comparing Δ9/actin values between 85-06 high stearate and TC plants, on average a 3 fold reduction in Δ9/actin was observed for the 85-06 plants. This data is graphed in Figure 26. Ranges in Δ9/actin ratios from 0.35 to 0.53, with an average of 0.43 were calculated for the RPA85-15 high stearate transgenics. In this experiment the average Δ9/actin ratio for the NT plants was 1.7. Comparing the average Δ9/actin ratio between NT controls and 85-15 high stearate plants, a 3.9 fold reduction in 85-15 Δ9 mRNA was demonstrated. An apparent 3 fold reduction in Δ9 mRNA level was observed for RPA85-15 high stearate transgenics when Δ9/actin ratios were compared between 85-15 high stearate and normal stearate (TC) plants. These data are graphed in Figure 27. These data indicate ribozyme-mediated reduction of Δ9-desaturase mRNA in transgenic plants expressing RPA85 ribozyme, and producing increased levels of stearic acid in the leaves.

Example 29: Evidence of Δ9 Desaturase Down Regulation in Maize Leaves as a Result of Active Ribozyme Activity

Plants were produced which were transformed with inactive versions of the Δ9 desaturase ribozyme genes. Data was presented demonstrating control levels of leaf stearate in the inactive Δ9 ribozyme transgenic lines RPA113-06 and 113-17. Ribozyme expression and northern analysis was performed for the RPA113-06 line. Δ9 desaturase protein levels were determined in plants of the RPA113-17 line. Ribozyme expression was measured as described herein. Plants 113-06-04, -07, and -10 expressed detectable levels of RPA113 inactive Δ9 ribozyme. Northern analysis was performed on 5 plants of the 113-06 line with leaf stearate ranging from 1.8 - 3.9 %, all of which fall within the range of controls. No reduction in Δ9 desaturase mRNA correlating with ribozyme expression or elevations in leaf stearate were found in the RPA113-06 plants as compared to controls, graphed in Figure 28. Protein analysis did not indicate any reduction in Δ9 desaturase protein levels correlating with elevated leaf stearate in the RPA113-17 plants. This data is graphed in Figure 29(a). Taken together, the data from the two RPA113 inactive transgenic lines indicate ribozyme activity is responsible for the high strearate phenotype observed in the RPA85 lines. The RPA85 ribozyme is the active version of the RPA113 ribozyme.

Example 30: Demonstration of Ribozyme Mediated Reduction in Stearoyl-ACP Δ9 Desaturase levels in Maize Leaves (RO) Δ9 Desaturase Levels in Maize Leaves (R0)

Part A Partial purification of stearoyl-ACP Δ9-desaturase from maize leaves. All procedures were performed at 4°C unless stated otherwise. Maize leaves (50 mg) were harvested and ground to a fine powder in liquid N_2 with a mortar and pestle. Proteins were extracted in one equal volume of Buffer A consisting of 25 mM sodium-phosphate pH 6.5, 1 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin, and 5 mM antipapin. The crude homogenate was centrifuged for 5 minutes at 10,000 x g. The supernatant was assayed for total protein concentration by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). One hundred micrograms of total protein was brought up to a final volume of 500 μl in Buffer A, added to 50 μl of mixed SP-sepharose beads (Pharmacia Biotech Inc., Piscataway, NJ), and resuspended by vortexing briefly. Proteins were allowed to bind to sepharose beads for 10 minutes while on ice. After binding, the Δ9 desaturase-sepharose material was centrifuged (10,000 x g) for 10 seconds, decanted, washed three times with Buffer A (500 μl), and washed one time with 200 mM sodium chloride (500 μl). Proteins were eluted by boiling in 50 μl of Treatment buffer (125 mM

Tris-Cl pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) for 5 mintues. Samples were centrifuged (10,000 x g) for 5 minutes. The supernatant was saved for Western anaylsis and the pellet consisting of sepharose beads was discarded.

Part B Western analysis method which was used to demonstrate reductions in stearoyl-ACP Δ9 desaturase. Partially purified proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels (10% PAGE) as described by Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of the head of phage T4, *Nature* 227, 660-685. To distinguish variation in Δ9 desaturase levels, included on each blot as a reference was purified and quantified overexpressed Δ9 desaturase from *E. coli* as described hereforth. Proteins were electrophoretically transferred to ECL™ nitrocellulose membranes (Amersham Life Sciences, Arlington Heights, Illinois) using a Pharmacia Semi-Dry Blotter (Pharmacia Biotech Inc., Piscataway, NJ), using Towbin buffer (Towbin *et al.* 1979). The nonspecific binding sites were blocked with 10% dry milk in phosphate buffer saline for 1 h. Immunoreactive polypeptides were detected using the ECL™ Western Blotting Detection Reagent (Amersham Life Sciences, Arlington Heights, Illinois) with rabbit antiserum raised against *E. coli* expressed maize Δ9 desaturase. The antibody was produced according to standard protocols by Berkeley Antibody Co. The secondary antibody was goat antirabbit serum conjugated to horseradish peroxidase (BioRad). Autoradiograms were scanned with a densitometer and quantified based on the relative amount of purified *E. coli* Δ9 desaturase. These experiments were duplicated and the mean reduction was recorded.

Part C Demonstration of Reductions in Δ9 desaturase levels in R0 maize leaves expressing ribozymes targeted to Δ9 desaturase mRNA. The high stearate transgenic line, RPA85-15, contains an intact copy of the fused multimer gene. Δ9 desaturase was partially purified from R0 maize leaves, using the protocol described herein. Western analysis was performed on ribozyme active (RPA85-15) and ribozyme inactive (RPA113-17) plants and nontransformed (HiII) plants as described above in part B. The natural variation of Δ9 desaturase was determined for the nontransformed line (HiII) by Western analysis see Figure 29 A. No reduction in Δ9 desaturase was observed with the ribozyme inactive line RPA113-17, all of which fell within the range as compared to the nontransformed line (HiII). An apparent 50% reduction of Δ9 desaturase was observed in six plants of line RPA85-15 (Figure 29 B) as compared with the controls. Concurrent with this, these same six plants also had increased stearate and reduced Δ9 desaturase mRNA (As described in Examples 28 and 32). However, nine active ribozyme plants

from line RPA85-15 did not have any significant reduction as compared with nontransformed line (HiII) and inactive ribozyme line (RPA113-17) (Figures 29 A and B). Collectively, these results suggest that the ribozyme activity in the six plants from line RPA85-15 is responsible for the reduced Δ9 desaturase.

Example 31: *E. coli* Expression and Purification of Maize Δ-9 desaturase enzyme

Part A The mature protein encoding portion of the maize Δ-9 desaturase cDNA was inserted into the bacterial T7 expression vector pET9D (Novagen Inc., Madison, WI). The mature protein encoding region was deduced from the mature castor bean polypeptide sequence. The alanine at position 32 (nts 239-241 of cDNA) was designated as the first residue. This is found within the sequence Ala.Val.Ala.Ser.Met.Thr. Restriction endonuclease *Nhe* I site was engineered into the maize sequence by PCR, modifying GCCTCC to GCTAGC and a *BamHI* site was added at the 3' end. This does not change the amino acid sequence of the protein. The cDNA sequence was cloned into pET9d vector using the Nhe I and Bam HI sites. The recombinant plasmid is designated as pDAB428. The maize Δ-9 desaturase protein expressed in bacteria has an additional methionine residue at the 5' end. This pDAB428 plasmid was transformed into the bacterial strain BL21 (Novagen, Inc., Madison, WI) and plated on LB/kanamycin plates (25 mg/ml). Colonies were resuspended in 10 ml LB with kanamycin (25 mg/ml) and IPTG (1mM) and were grown in a shaker for 3 hours at 37°C. The cells were harvested by centrifugation at 1000xg at 4°C for 10 minutes. The cells were lysed by freezing and thawing the cell pellet 2X, followed by the addition of 1 ml lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1 % Triton X100, 100 ug/ml DNAse I, 100 ug/ml RNAse A, and 1 mg/ml lysozyme). The mixture was incubated for 15 minutes at 37°C and then centrifuged at 1000 Xg for 10 minutes at 4°C. The supernatant is used as the soluble protein fraction.

The supernatant, adjusted to 25 mM sodium phosphate buffer (pH 6.0), was chilled on ice for 1 hr. Afterwards, the resulting flocculant precipitant was removed by centrifugation. The ice incubation step was repeated twice more after which the solution remained clear. The clarified solution was loaded onto a Mono S HR 10/10 column (Pharmacia) that had been equilibrated in 25 mM sodium phosphate buffer (pH 6.0). Basic proteins bound to the column matrix were eluted using a 0-500 mM NaCl gradient over 1 hr (2 ml/min; 2 ml fractions). The putative protein of interest was subjected to SDS-PAGE, blotted onto PVDF membrane, visualized with coomassie blue, excised, and sent to Harvard Microchem for amino-terminal sequence analysis. Comparison of the

protein's amino terminal sequence to that encoded by the cDNA clone revealed that the protein was indeed Δ 9. Spectrophotometric analysis of the diiron-oxo component associated with the expressed protein (Fox et al., 1993 *Proc. Natl. Acad. Sci. USA*. 90. 2486-2490), as well as identification using a specific nonheme iron stain (Leong et al., 1992 *Anal. Biochem*. 207, 317-320) confirmed that the purified protein was Δ-9.

Part B Production of polyclonal antiserum

The *E. coli* produced Δ-9 protein, as determined by amino terminal sequencing, was gel purified via SDS-PAGE, excised, and sent in the gel matrix to Berkeley Antibody Co., Richmond, CA, for production of polyclonal sera in rabbits. Titers of the antibodies against Δ-9 were performed via western analysis using the ECL Detection system (Amersham, Inc.)

Part C Purification of Δ9 desaturase from corn kernels

Protein Precipitation: Δ9 was purified from corn kernels following homogenization using a Warring blender in 25 mM sodium phosphate buffer (pH 7.0) containing 25 mM sodium bisulfite and a 2.5% polyvinylpolypyrrolidone. The crude homogenate was filtered through cheesecloth, centrifuged (10,000xg) for 0.25 h and the resulting supernatant was filtered once more through cheesecloth. In some cases, the supernatant was fractionated via saturated ammonium sulfate precipitation by precipitation at 20% v/v followed by 80% v/v. Extracts obtained from high oil germplasm were fractionated by adding a 50% polyethylene glycol solution (mw=8000) at final concentrations of 5- and 25% v/v. In all cases, the Δ9 protein precipitated at either 80% ammonium sulfate or 25% polyethylene glycol. The resulting pellets were then dialyzed extensively in 25mM sodium phosphate buffer (pH 6.0).

Cation Exchange Chromotography: The solubilized pellet material described above was clarified via centrifugation and applied to Mono S HR10/10 column equilibrated in 25 mM sodium phosphate buffer (pH 6.0). After extensive column washing, basic proteins bound to the column matrix were eluted using a 0-500 mM NaCl gradient over 1 hr (2 ml/min: 2 ml fractions). Typically, the Δ9 protein eluted between 260-and 350 mM NaCl., as determined by enzymatic and western analysis. After dialysis, this material was further fracionated by acyl carrier protein (ACP)- sepharose and phenyl superose chromatography.

Acyl Carrier Protein-Sepharose Chromatography: ACP was purchased from Sigma Chemical Company and purified via precipitation at pH 4.1 (Rock and Cronan, 1981 *J. Biol. Chem.* 254, 7116-7122) before linkage to the beads. ACP-sepharose was prepared by covalently binding 100 mg of ACP to cyanogen bromide activated sepharose 4B beads, essentially as described by Pharmacia, Inc., in the package insert. After linkage and blocking of the remaining sites with glycine, the ACP-sepharose material was packed into a HR 5/5 column (Pharmacia, Inc.) and equilibrated in 25 mM sodium phosphate buffer (pH 7.0). The dialyzed fractions identified above were then loaded onto the column (McKeon and Stumpf, 1982 *J. Biol. Chem.* 257, 12141-12147; Thompson *et al.*, 1991 *Proc. Natl. Acad. Sci.* USA 88, 2578-2582). After extensive column washing, ACP-binding proteins were eluted using 1 M NaCl. Enzymatic and western analysis, followed by amino terminal sequencing, indicated that the eluent contained Δ-9 protein. The Δ-9 protein purified from corn was determined to have a molecular size of approximately 38 kDa by SDS-PAGE analysis (Hames, 1981 in Gel Electrophoresis of Proteins: A Practical Approach , eds Hames BD and Rickwood, D. , IRL Press, Oxford).

Phenyl Sepharose Chromatography: The fractions containing Δ9 obtained from the ACP-Sepharose column were adjusted to 0.4 M ammonium sulfate (25 mM sodium phosphate, pH 7.0) and loaded onto a Pharmacia Phenyl Superose column (HR 10/10). Proteins were eluted by running a gradient (0.4 - 0.0 M ammonium sulfate) at 2 ml/min for 1 hour. The Δ9 protein typically eluted between 60- and 30 mM ammonium sulfate as determined by enzymatic and western analysis.

<u>Example 32: Evidence for the Increase in Stearic Acid in Leaves as a Result of Transformation of Plants with Δ9 Desaturase Ribozymes</u>

Part A Method used to determine the stearic acid levels in plant tissues. The procedure for extraction and esterification of fatty acids from plant tissue was modified from a described procedure (Browse *et. al.*, 1986, Anal. Biochem. 152, 141-145). One to 20 mg of plant tissue was placed in Pyrex 13 mm screw top test tubes. After addition of 1 ml of methanolic HCL (Supelco, Bellefonte, PA), the tubes were purged with nitrogen gas and sealed. The tubes were heated at 80°C for 1 hour and allowed to cool. The heating in the presence of the methanolic HCL results in the extraction as well as the esterification of the fatty acids. The fatty acid methyl esters were removed from the reaction mixture by extraction with hexane. One ml of hexane and 1 ml of 0.9% (w/v) NaCl was added followed by vigorous shaking of the test tubes. After centrifugation of the tubes at 2000 rpm for 5 minutes the top hexane layer was removed and used for fatty acid methyl ester

analysis. Gas chromatograph analysis was performed by injection of 1 μl of the sample on a Hewlett Packard (Wilmington, DE) Series II model 5890 gas chromatograph equipped with a flame ionization detector and a J&W Scientific (Folsom, CA) DB-23 column. The oven temperature was 150°C throughout the run and the flow of the carrier gas (helium) was 80 cm/sec. The run time was 20 minutes. The conditions allowed for the separation of the 5 fatty acid methyl esters of interest: C16:0, palmityl methyl ester; C18:0, stearyl methyl ester; C18:1, oleoyl methyl ester; C18:2, linoleoyl methyl ester; and C18:3, linolenyl methyl ester. Data collection and analysis was performed with a Hewlett Packard Series II Model 3396 integrator and a PE Nelson (Perkin Elmer, Norwalk, CT) data collection system. The percentage of each fatty acid in the sample was taken directly from the readouts of the data collection system. Quantitative amounts of each fatty acid were calculated using the peak areas of a standard (Matreya, Pleasant Gap, PA) which consisted of a known amount of the five fatty acid methyl esters. The amount calculated was used to estimate the percentage, of total fresh weight, represented by the five fatty acids in the sample. An adjustment was not made for loss of fatty acids during the extraction and esterification procedure. Recovery of the standard sample, after subjecting it to the extraction and esterification procedure (with no tissue present), ranged from 90 to 100% depending on the original amount of the sample. The presence of plant tissue in the extraction mixture had no effect on the recovery of the known amount of standard.

Part B Demonstration of an increase in stearic acid in leaves due to introduction of Δ9 desaturase ribozymes. Leaf tissue from individual plants was assayed for stearic acid as described in Part A. A total of 428 plants were assayed from 35 lines transformed with active Δ9 desaturase ribozymes (RPA85, RPA114, RPA118) and 406 plants from 31 lines transformed with Δ9 desaturase inactive ribozymes (RPA113, RPA115, RPA119). Table XI summarizes the results obtained for stearic acid levels in these plants. Seven percent of the plants from the active lines had stearic acid levels greater than 3%, and 2% had levels greater than 5%. Only 3% of the plants from the inactive lines had stearic acid levels greater than 3%. Two percent of the control plants had leaves with stearate greater than 3%. The controls included 49 non-transformed plants and 73 plants transformed with a gene not related to Δ9 desaturase. There were no plants from the inactive lines or controls that had leaf stearate greater than 4%. Two of the lines transformed with the active Δ9 desaturase ribozyme RPA85 produced many plants which exhibited increased stearate in their leaves. Line RPA85-06 had 6 out of the 15 plants assayed with stearic acid levels which were between 3 and 4 %, about 2-fold greater than the average of the

controls (Figure 30) The average stearic acid content of the control plants (122 plants) was 1.69% (SD+/-0.49%). The average stearic acid content of leaves from line RPA85-06 was 2.86% (+/-0.57%). Line RPA85-15 had 6 out of 15 plants assayed with stearic acid levels which were approximately 4-fold greater than the average of the controls (Figure 31). The average leaf stearic acid content of line RPA85-15 was 3.83% (+/-2.53%). When the leaf analysis was repeated for RPA85-15 plants, the stearic acid level in leaves from plants previously shown to have normal stearic acid levels remained normal and leaves from plants with high stearic acid were again found to be high (Figure 31). The stearic acid levels in leaves of plants from two lines which were transformed with an inactive Δ9 desaturase ribozyme, RPA113, is shown in Figures 32 and 33. RPA113-06 had three plants with a stearic acid content of 3% or higher. The average stearic acid content of leaves from line RPA113-06 was 2.26% (+/-0.65%). RPA 113-17 had no plants with leaf stearic acid content greater than 3%. The average stearic acid content of leaves from line RPA113-17 was 1.76% (+/-0.29%). The stearic acid content of leaves from 15 control plants is shown in Figure 34. The average stearic acid content for these 15 control plants was 1.70% (+/-0.6%). When compared to the control and inactive Δ9 desaturase ribozyme data, the results obtained for stearic acid content in RPA85-06 and RPA85-15 demonstrate an increase in stearic acid content due to the introduction of the Δ9 desaturase ribozyme.

Example 33: Inheritance of the High Stearic Acid Trait in Leaves

Part A Results obtained with stearic acid levels in leaves from offspring of high stearic acid plants. Plants from line RPA85-15 were pollinated as described herein. Twenty days after pollination zygotic embryos were excised from immature kernels from these RPA85-15 plants and placed in a tube on media as described herein for growth of regenerated plantlets. After the plants were transferred to the greenhouse, fatty acid analysis was performed on the leaf tissue. Figure 35 shows the stearic acid levels of leaves from 10 different plants for one of the crosses, RPA85-15.07 selfed. Fifty percent of the plants had high leaf stearic acid and 50% had normal leaf stearic acid. Table XII shows the results from 5 different crosses of RPA85-15 plants. The number of plants with high stearic acid ranged from 20 to 50%.

Part B Results demonstrating reductions in Δ9 desaturase levels in next generation (R1) maize leaves expressing ribozymes targeted to Δ9 desaturase mRNA. In next generation maize plants that showed a high stearate content (see above Part A), Δ9 desaturase was

partially purified from R1 maize leaves, using the protocol described herein. Western analysis was performed on several of the high stearate plants. In leaves of next generation plants, a 40-50% reduction of Δ9 desaturase was observed in those plants that had high stearate content (Figure 36). The reduction was comparable to R0 maize leaves. This reduction was observed in either OQ414 plants crossed with RPA85-15 pollen or RPA85-15 plants crossed with self or siblings. Therefore, this suggests that the gene encoding the ribozyme is heritable.

Example 34: Increase in Stearic Acid in Plant Tissues Using Antisense- Δ9 Desaturase

Part A Method for culturing somatic embryos of maize. The production and regeneration of maize embryogenic callus has been described herein. Somatic embryos make up a large part of this embryogenic callus. The somatic embryos continued to form in callus because the callus was transferred every two weeks. The somatic embryos in embryogenic callus continued to proliferate but usually remained in an early stage of embryo development because of the 2,4-D in the culture medium. The somatic embryos regenerated into plantlets because the callus was subjected to a regeneration procedure described herein. During regeneration the somatic embryo formed a root and a shoot, and ceases development as an embryo. Somatic embryos were made to develop as seed embryos, i.e., beyond the early stage of development found in embryogenic callus and no regeneration, by a specific medium treatment. This medium treatment involved transfer of the embryogenic callus to a Murashige and Skoog medium (MS; described by Murashige and Skoog in 1962) which contains 6% (w/v) sucrose and no plant hormones. The callus was grown on the MS medium with 6% sucrose for 7 days and then the somatic embryos were individually transferred to MS medium with 6% sucrose and 10 µM abscisic acid (ABA). The somatic embryos were assayed for fatty acid composition as described herein after 3 to 7 days of growth on the ABA medium. The fatty acid composition of somatic embryos grown on the above media was compared to the fatty acid composition of embryogenic callus and maize zygotic embryos 12 days after pollination (Table XIII). The fatty acid composition of the somatic embryos was different than that of the embryogenic callus. The embryogenic callus had a higher percentage of C16:0 and C18:3, and a lower percentage of C18:1 and C18:2. The percentage of lipid represented by the fresh weight was different for the embryogenic callus when compared to the somatic embryos; 0.4% versus 4.0%. The fatty acid composition of the zygotic embryos and somatic embryos were very similar and their percentage of lipid represented by the fresh weight were nearly identical. It was

concluded that the somatic embryo culture system described above would be an useful *in vitro* system for testing the effect of certain genes on lipid synthesis in developing embryos of maize.

Part B Increase in stearic acid in somatic embryos of maize as a result of the introduction of an antisense- Δ9 desaturase gene. Somatic embryos were produced using the method described herein from embryogenic callus transformed with pDAB308/pDAB430. The somatic embryos from 16 different lines were assayed for fatty acid composition. Two lines, 308/430-12 and 308/430-15, were found to produce somatic embryos with high levels of stearic acid. The stearic acid content of somatic embryos from these two lines is compared to the stearic acid content of somatic embryos from their control lines in Figures 37 and 38. The control lines were from the same culture that the transformed lines came from except that they were not transformed. For line 308/430-12, stearic acid in somatic embryos ranged from 1 to 23% while the controls ranged from 0.5 to 3%. For line 308/430-15, stearic acid in somatic embryos ranged from 2 to 15% while the controls ranged from 0.5 to 3%. More than 50% of the somatic embryos had stearic acid levels which were above the range of the controls in both the transformed lines. The above results indicate that an antisense- Δ9 desaturase gene can be used to raise the stearic acid levels in somatic embryos of maize.

Part C Demonstration of an increase in stearic acid in leaves due to introduction of an antisense- Δ9 desaturase gene. Embryogenic cultures from lines 308/430-12 and 308/430-15 were used to regenerate plants. Leaves from these plants were analyzed for fatty acid composition using the method previously described. Only 4 plants were obtained from the 308/430-15 culture and the stearic acid level in the leaves of these plants were normal, 1-2%. The stearic acid levels in leaves from plants of line 308/430-12 are shown in Figure 39. The stearic acid levels in leaves ranged from 1 to 13% in plants from line 308/430-12. About 30% of the plants from line 308/430-12 had stearic acid levels above the range observed in the controls, 1-2%. These results indicate that the stearic acid levels can be raised in leaves of maize by introduction of an antisense- Δ9 desaturase gene.

By "antisense" is meant a non-enzymatic nucleic acid molecule that binds to a RNA (target RNA) by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm et al., 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review see Stein and Cheng, 1993 *Science* 261, 1004).

Example 35: Amylose Content Assay of Maize Pooled Starch Sample and Single Kernel

The amylose content was assayed by the method of Hovenkamp-Hermelink et al. (Potato Research 31:241-246) with modifications. For pooled starch sample, 10 mg to 100 mg starch was dissolved in 5 ml 45% perchloric acid in plastic culture tube. The solution was mixed occasionally by vortexing. After one hour, 0.2 ml of the starch solution was diluted to 10 ml by H_2O. 0.4 ml of the diluted solution was then mixed with 0.5 ml diluted Lugol's solution (Sigma) in 1 ml cuvet. Readings at 618 nm and 550 nm were immediately taken and the R ratio (618 nm/550 nm) was calculated. Using standard equation P (percentage of amylose) = (4.5R-2.6)/(7.3-3R) generated from potato amylose and maize amylopectin (Sigma, St. Louis), amylose content was determined. For frozen single kernel sample, same procedure as above was used except it was extracted in 45% perchloric acid for 20 min instead for one hour.

Example 36: Starch Purification and Granular Bound Starch Synthase (GBSS) Assay

The purification of starch and following GBSS activity assay were modified from the methods of Shure et al. (Cell, 35:225-233, 1983) and Nelson et al. (Plant Physiology, 62:383-386, 1978). Maize kernel was homogenized in 2 volume (v/w) of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and filtrated through 120 µm nylon membrane. The material was then centrifuged at 5000 g for 2 min and the supernatant was discarded. The pellet was washed three times by resuspending in water and removing supernatant by centrifugation. After washing, the starch was filtrated through 20 µm nylon membrane and centrifuged. Pellet was then lyophilized and stored in - 20 °C until used for activity assay.

A standard GBSS reaction mixture contained 0.2 M Tricine, pH 8.5, 25 mM Glutathione, 5 mM EDTA, 1 mM ^{14}C ADPG (6 nci/µmol), and 10 mg starch in a total volume of 200 µl. Reactions were conducted at 37 °C for 5 min and terminated by adding 200 µl of 70% ethanol (v/v) in 0.1 M KCl. The material was centrifuged and unincorporated ADPG in the supernatant is removed. The pellet was then washed four time with 1ml water each in the same fashion. After washing, pellet was suspended in 500 µl water, placed into scintillation vial, and the incorporated ADPG was counted by a Beckman (Fullerton, CA) scintillation counter. Specific activity was given as pmoles of ADPG incorporated into starch per min per mg starch.

Example 37: Analysis of Antisense-GBSS Plants

Because of the segregation of R2 seeds, single kernels should therefore be analyzed for amylose content to identify phenotype. Because of the large amount of samples generated in this study, a two-step screening strategy was used. In the first step, 30 kernels were taken randomly from the same ear, freeze-dried and homogenized into starch flour. Amylose assays on the starch flours were carried out. Lines with reduced amylose content were identified by statistical analysis. In the second step, amylose content of the single kernels in the lines with reduced amylose content was further analyzed (25 to 50 kernels per ear). Two sets of controls were used in the screening, one of the sets were untransformed lines with the same genetic background and the other were transformed lines which did not carry transgene due to segregation (Southern analysis negative line).

81 lines representing 16 transformation events were examined at the pooled starch level. Among those lines, six with significant reduction of amylose content by statistical analysis were identified for further single kernel analysis. One line, 308/425-12.2.1, showed significant reduction of amylose content (Figure 40).

Twenty five individual kernels of CQ806, a conventional maize inbred line, were analyzed. The amylose content of CQ806 ranged from 24.4% to 32.2%, averaging 29.1%. The single kernel distribution of amylose content is skewed slightly towards lower amylose contents. Forty nine single kernels of 308/425-12.2.1.1 were analyzed. Given that 308/425-12.2.1.1 resulted from self pollination of a hemizygous individual, the expected distribution would consist of 4 distinct genetic classes present in equal frequencies since endosperm is a triploid tissue. The 4 genetic classes consist of individuals carrying 0, 1, 2, and 3 copies of the antisense construct. If there is a large dosage effect for the transgene, then the distribution of amylose contents would be tetramodal. One of the modes of the resulting distribution should be indistinguishable from the non-transgenic parent. If there is no dosage effect for the transgene (individuals carrying 1, 2 or 3 copies of the transgene are phenotypically equivalent), then the distribution should be bimodal with one of the modes identical to the parent. The number of individuals included in the modes should be 3:1 of transgenic:parental. The distribution for 308/425-12.2.1.1 is distinctly trimodal. The central mode is approximately twice the size of either other mode. The two distal modes are of approximately equal size. Goodness of fit to a 1:2:1 ratio was tested and the fit was excellent.

Further evidence was available demonstrating that the mode with the highest amylose content was identical to the non-transgenic parent. This was done using discriminant analysis. The CQ806 and 308/425-12.2.1.1 data sets were combined for this analysis. The distance metrics used in the analysis were calculated using amylose contents only. The estimates of variance from the individual analyses were used in all tests. No pooled estimate of variance was employed. The original data was tested for reclassification. Based on the discriminant analysis, the entire mode of the 308/425-12.2.1.1 distribution with the highest amylose content would be more appropriately classified as parental. This is strong confirmation that this mode of the distribution is parental. Of the remaining two modes, the central mode is approximately twice the size of the lowest amylose content mode. This would be expected if the central mode includes two genetic classes: individuals with 1 or 2 copies of the antisense construct. The mode with the lowest amylose content thus represents those individuals which are fully homozygous (3 copies) for the antisense construct. The 2:1 ratio was tested and could not be rejected on the basis of the data.

This analysis indicates that the antisense GBSS gene as functioning in 308/425-12.2.1.1 demonstrates a dosage dependent reduction in amylose content of maize kernels.

Example 38: Analysis of Ribozyme-GBSS Plants

The same two-step screening strategy as in the antisense study (Example 37) was used to analyze ribozyme-GBSS plants. 160 lines representing 11 transformation events were examined in the pooled starch level. Among the control lines (both untransformed line and Southern negative line), the amylose content varied from 28% to 19%. No significant reduction was observed among all lines carrying ribozyme gene (Southern positive line). More than 20 selected lines were further analyzed in the single kernel level, no significant amylose reduction as well as segregation pattern were found. It was apparent that ribozyme did not cause any alternation in the phenotypic level.

Transformed lines were further examined by their GBSS activity (as described in Example 36). For each line, 30 kernels were taken from the frozen ear and starch was purified. Table XIV shows the results of 9 plants representing one transformation event of the GBSS activity in the pooled starch samples, amylose content in the pooled starch samples, and Southern analysis results. Three southern negative lines: RPA63.0283, RPA63.0236, and RPA63.0219 were used as control.

The GBSS activities of control lines RPA63.0283, RPA63.0236, and RPA63.0219 were around 300 units/mg starch. In lines RPA63.0211, RPA63.0218, RPA63.0209, and RPA63.0210, a reduction of GBSS activity to more than 30% was observed. The correlation of varied GBSS activity to the Southern analysis in this group (from RPA63.0314 to RPA63.0210 of Table XIV) indicated that the reduced GBSS activity was caused by the expression of ribozyme gene incorporated into the maize genome.

GBSS activities at the single kernel level of line RPA 63.0218 (Southern positive and reduced GBSS activity in pooled starch) was further examined, using RPA63.0306 (Southern negative and GBSS activity normal in pooled starch) as control. About 30 kernels from each line were taken, and starch samples were purified from each kernel individually. Figure 41 clearly indicated reduced GBSS activity in line RPA63.0218 compared to RPA63.0306.

Other embodiments are within the following claims.

TABLE 1

Characteristics of naturally occurring ribozymes

Group I Introns

- Size: ~150 to >1000 nucleotides.
- Requires a U in the target sequence immediately 5' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site.
- Reaction mechanism: attack by the 3'-OH of guanosine to generate cleavage products with 3'-OH and 5'-guanosine.
- Additional protein cofactors required in some cases to help folding and maintainance of the active structure [1].
- Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.
- Major structural features largely established through phylogenetic comparisons, mutagenesis, and biochemical studies [2,3].
- Complete kinetic framework established for one ribozyme [4,5,6,7].
- Studies of ribozyme folding and substrate docking underway [8,9,10].
- Chemical modification investigation of important residues well established [11,12].
- The small (4-6 nt) binding site may make this ribozyme too non-specific for targeted RNA cleavage, however, the Tetrahymena group I intron has been used to repair a "defective" b-galactosidase message by the ligation of new b-galactosidase sequences onto the defective message [13].

RNAse P RNA (M1 RNA)

- Size: ~290 to 400 nucleotides.
- RNA portion of a ubiquitous ribonucleoprotein enzyme.
- Cleaves tRNA precursors to form mature tRNA [14].
- Reaction mechanism: possible attack by M^{2+}-OH to generate cleavage products with 3'-OH and 5'-phosphate.
- RNAse P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.
- Recruitment of endogenous RNAse P for therapeutic applications is possible through hybridization of an External Guide Sequence (EGS) to the target RNA [15,16]
- Important phosphate and 2' OH contacts recently identified [17,18]

Group II Introns

- Size: >1000 nucleotides.
- Trans cleavage of target RNAs recently demonstrated [19,20].
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.
- Only natural ribozyme with demonstrated participation in DNA cleavage [21,22] in addition to RNA cleavage and ligation.

  Major structural features largely established through phylogenetic comparisons [23].

Table I

Important 2' OH contacts beginning to be identified [24]
- Kinetic framework under development [25]

Neurospora VS RNA

- Size: ~144 nucleotides.
- Trans cleavage of hairpin target RNAs recently demonstrated [26].
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- Only 1 known member of this class. Found in Neurospora VS RNA.

Hammerhead Ribozyme
(see text for references)
- Size: ~13 to 40 nucleotides.
- Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.
- Essential structural features largely defined, including 2 crystal structures []
- Minimal ligation activity demonstrated (for engineering through *in vitro* selection) []
- Complete kinetic framework established for two or more ribozymes [].
- Chemical modification investigation of important residues well established [].

Hairpin Ribozyme

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.
- Essential structural features largely defined [27,28,29,30]
- Ligation activity (in addition to cleavage activity) makes ribozyme amenable to engineering through *in vitro* selection [31]
- Complete kinetic framework established for one ribozyme [32].
- Chemical modification investigation of important residues begun [33,34].

Hepatitis Delta Virus (HDV) Ribozyme

- Size: ~60 nucleotides.
- Trans cleavage f target RNAs demonstrated [35].
- Binding sites and structural requirements not fully determined. although no sequences 5' of cleavage site are required. Folded ribozyme contains a pseud knot structure [36].

Table I

Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.

- Only 2 known members of this class. Found in human HDV.
- Circular form of HDV is active and shows increased nuclease stability [37]

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Table I

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Table II

Tabl II: 2.5 μm I RNA Synth sis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 μL	2.5
S-Ethyl Tetrazole	23.8	238 μL	2.5
Acetic Anhydride	100	233 μL	5 sec
N-Methyl Imidazole	186	233 μL	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery.

Table IIIA: GBSS Hammerhead Substrate Sequence

nt. Position	Substrate	Seq. ID No.	nt. Position	Substrate	Seq. ID No.
12	CGAUCGAUC GCCACAGC	26	538	GGUCGUCUC UCCCCGCU	27
68	GAAGGAAUA AACUCACU	28	540	UCGUCUCUC CCCGCUAC	29
73	AAUAAACUC ACUGCCAG	30	547	UCCCCGCUA CGACCAGU	31
103	AGAAGUGUA CUGCUCCG	32	556	CGACCAGUA CAAGGACG	33
109	GUACUGCUC CGUCCACC	34	581	ACCAGCGUC GUGUCCGA	35
113	UGCUCCGUC CACCAGUG	36	586	CGUCGUGUC CGAGAUCA	37
146	GGGCUGCUC AUCUCGUC	38	593	UCCGAGAUC AAGAUGGG	39
149	CUGCUCAUC UCGUCGAC	40	610	AGACAGGUA CGAGACGG	41
151	GCUCAUCUC GUCGACGA	42	620	GAGACGGUC AGGUUCUU	43
154	CAUCUCGUC GACGACCA	44	625	GGUCAGGUU CUUCCACU	45
169	CAGUGGAUU AAUCGGCA	46	626	GUCAGGUUC UUCCACUG	47
170	AGUGGAUUA AUCGGCAU	48	628	CAGGUUCUU CCACUGCU	49
173	GGAUUAAUC GGCAUGGC	50	629	AGGUUCUUC CACUGCUA	51
186	UGGCGGCUC UAGCCACG	52	637	~~CCACUGCUA CAAGGCGG~~	53
188	GCGGCUCUA GCCACGUC	54	661	CCGCGUGUU CGUUGACC	55
196	AGCCACGUC GCAGCUCG	56	662	CGCGUGUUC GUUGACCA	57
203	UCGCAGCUC GUCGCAAC	58	665	GUGUUCGUU GACCACCC	59
206	CAGCUCGUC GCAACGCG	60	679	CCCACUGUU CCUGGAGA	61
230	CUGGGCGUC CCGGACGC	62	680	CCACUGUUC CUGGAGAG	63
241	GGACGCGUC CACGUUCC	64	692	GAGAGGGUU UGGGGAAA	65
247	GUCCACGUU CCGCCGCG	66	693	AGAGGGUUU GGGGAAAG	67
248	UCCACGUUC CGCCGCGG	68	716	GAGAAGAUC UACGGGCC	69
292	GACGGCGUC GGCGGCGG	70	718	GAAGAUCUA CGGGCCUG	71
308	GACACGCUC AGCAUUCG	72	742	AACGGACUA CAGGGACA	73
314	CUCAGCAUU CGGACCAG	74	763	GCUGCGGUU CAGCCUGC	75
315	UCAGCAUUC GGACCAGC	76	764	CUGCGGUUC AGCCUGCU	77
344	CCCAGGCUC CAGCACCA	78	773	AGCCUGCUA UGCCAGGC	79
385	GGCCAGGUU CCCGUCGC	80	788	GCAGCACUU GAAGCUCC	81
386	GCCAGGUUC CCGUCGCU	82	795	UUGAAGCUC CAAGGAUC	83
391	GUUCCCGUC GCUCGUCG	84	803	CCAAGGAUC CUGAGCCU	85
395	CCGUCGCUC GUCGUGUG	86	812	CUGAGCCUC AACAACAA	87
398	UCGCUCGUC GUGUGCGC	88	826	CAACCCAUA CUUCUCCG	89
425	AUGAACGUC GUCUUCGU	90	829	CCCAUACUU CUCCGGAC	91
428	AACGUCGUC UUCGUCGG	92	830	CCAUACUUC UCCGGACC	93
430	CGUCGUCUU CGUCGGCG	94	832	AUACUUCUC CGGACCAU	95
431	GUCGUCUUC GUCGGCGC	96	841	~~CGGACCAUA CGGGGAGG~~	97
434	GUCUUCGUC GGCGCCGA	98	854	GAGGACGUC GUGUUCGU	99
473	GGCGGCCUC GGCGACGU	100	859	CGUCGUGUU CGUCUGCA	101
482	GGCGACGUC CUCGGCGG	102	860	GUCGUGUUC GUCUGCAA	103
485	GACGUCCUC GGCGGCCU	104	863	GUGUUCGUC UGCAACGA	105
527	CACCGUGUC AUGGUCGU	106	888	CCGGCCCUC UCUCGUGC	107
533	GUCAUGGUC GUCUCUCC	108	890	GGCCCUCUC UCGUGCUA	109
536	AUGGUCGUC UCUCCCCG	110	892	CCCUCUCUC GUGCUACC	111
898	CUCGUGCUA CCUCAAGA	112	1241	AUGGACGUC AGCGAGUG	113
902	UGCUACCUC AAGAGCAA	114	1270	GGACAAGUA CAUCGCCG	115
913	GAGCAACUA CCAGUCCC	116	1274	AAGUACAUC GCCGUGAA	117
919	CUACCAGUC CCACGGCA	118	1285	CGUGAAGUA C ACGUGU	119
929	CACGGCAUC UACAGGGA	120	1294	CGACGUGUC GACGGCCG	121
931	CGGCAUCUA CAGGGACG	122	1346	GCGGAGGUC GGGCUCCC	123
951	AGACCGCUU UCUGCAUC	124	1352	GUCGGGCUC CCGGUGGA	125
952	GACCGCUUU CUGCAUCC	126	1370	CGGAACAUC CCGCUGGU	127
953	ACCGCUUUC UGCAUCCA	128	1384	GGUGGCGUU CAUCGGCA	129

Table IIIA

nt. Position	Substrate	Seq. ID No.	nt. Position	Substrate	Seq. ID No.
959	UUCUGCAUC CACAACAU	130	1385	GUGGCGUUC AUCGGCAG	131
968	CACAACAUC UCCUACCA	132	1388	GCGUUCAUC GGCAGGCU	133
970	CAACAUCUC CUACCAGG	134	1421	CCCGACGUC AUGGCGGC	135
973	CAUCUCCUA CCAGGGCC	136	1436	GCCGCCAUC CCGCAGCU	137
985	GGGCCGGUU CGCCUUCU	138	1445	CCGCAGCUC AUGGAGAU	139
986	GGCCGGUUC GCCUUCUC	140	1472	GUGCAGAUC GUUCUGCU	141
991	GUUCGCCUU CUCCGACU	142	1475	CAGAUCGUU CUGCUGGG	143
992	UUCGCCUUC UCCGACUA	144	1476	AGAUCGUUC UGCUGGGC	145
994	CGCCUUCUC CGACUACC	146	1501	GAAGAAGUU CGAGCGCA	147
1000	CUCCGACUA CCCGGAGC	148	1502	AAGAAGUUC GAGCGCAU	149
1016	CUGAACCUC CCGGAGAG	150	1514	CGCAUGCUC AUGAGCGC	151
1027	GGAGAGAUU CAAGUCGU	152	1534	GGAGAAGUU CCCAGGCA	153
1028	GAGAGAUUC AAGUCGUC	154	1535	GAGAAGUUC CCAGGCAA	155
1033	AUUCAAGUC GUCCUUCG	156	1559	GCCGUGGUC AAGUUCAA	157
1036	CAAGUCGUC CUUCGAUU	158	1564	GGUCAAGUU CAACGCGG	159
1039	GUCGUCCUU CGAUUUCA	160	1565	GUCAAGUUC AACGCGGC	161
1040	UCGUCCUUC GAUUUCAU	162	1589	CACCACAUC AUGGCCGG	163
1044	CCUUCGAUU UCAUCGAC	164	1610	GACGUGCUC GCCGUCAC	165
1045	CUUCGAUUU CAUCGACG	166	1616	CUCGCCGUC ACCAGCCG	167
1046	UUCGAUUUC AUCGACGG	168	1627	CAGCCGCUU CGAGCCCU	169
1049	GAUUUCAUC GACGGCUA	170	1628	AGCCGCUUC GAGCCCUG	171
1057	CGACGGCUA CGAGAAGC	172	1643	UGCGGCCUC AUCCAGCU	173
1085	CGGAAGAUC AACUGGAU	174	1646	GGCCUCAUC CAGCUGCA	175
1106	GCCGGGAUC CUCGAGGC	176	1666	GAUGCGAUA CGGAACGC	177
1109	GGGAUCCUC GAGGCCGA	178	1690	CUGCGCGUC CACCGGUG	179
1124	GACAGGGUC CUCACCGU	180	1703	GGUGGACUC GUCGACAC	181
1127	AGGGUCCUC ACCGUCAG	182	1706	GGACUCGUC GACACCAU	183
1133	CUCACCGUC AGCCCCUA	184	1715	GACACCAUC AUCGAAGG	185
1141	CAGCCCCUA CUACGCCG	186	1718	ACCAUCAUC GAAGGCAA	187
1144	CCCCUACUA CGCCGAGG	188	1735	GACCGGGUU CCACAUGG	189
1157	GAGGAGCUC AUCUCCGG	190	1736	ACCGGGUUC CACAUGGG	191
1160	GAGCUCAUC UCCGGCAU	192	1751	GGCCGCCUC AGCGUCGA	193
1162	GCUCAUCUC CGGCAUCG	194	1757	CUCAGCGUC GACUGCAA	195
1169	UCCGGCAUC GCCAGGGG	196	1769	UGCAACGUC GUGGAGCC	197
1187	UGCGAGCUC GACAACAU	198	1787	GCGGACGUC AAGAAGGU	199
1196	GACAACAUC AUGCGCCU	200	1807	CACCACCUU GCAGCGCG	201
1205	AUGCGCCUC ACCGGCAU	202	1820	CGCGCCAUC AAGGUGGU	203
1214	ACCGGCAUC ACCGGCAU	204	1829	AAGGUGGUC GGCACGCC	205
1223	ACCGGCAUC GUCAACGG	206	1843	GCCGGCGUA CGAGGAGA	207
1226	GGCAUCGUC AACGGCAU	208	1871	UGCAUGAUC CAGGAUCU	209

Table IIIA

nt. Position	Substrate	Seq. ID No.
1878	UCCAGGAUC UCUCCUGG	210
1880	CAGGAUCUC UCCUGGAA	212
1882	GGAUCUCUC CUGGAAGG	214
1922	GUGCUGCUC AGCCUCGG	216
1928	CUCAGCCUC GGGGUCGC	218
1934	CUCGGGGUC GCCGGCGG	220
1955	CCAGGGGUC GAAGGCGA	222
1970	GAGGAGAUC GCGCCGCU	224
1979	GCGCCGCUC GCCAAGGA	226
2012	UGAAGAGUU CGGCCUGC	227
2013	GAAGAGUUC GGCCUGCA	228
2033	CCCCUGAUC UCGCGCGU	229
2035	CCUGAUCUC GCGCGUGG	230
2055	AAACAUGUU GGGACAUC	231
2063	UGGGACAUC UUCUUAUA	232
2065	GGACAUCUU CUUAUAUA	233
2066	GACAUCUUC UUAUAUAU	234
2068	CAUCUUCUU AUAUAUGC	235
2069	AUCUUCUUA UAUAUGCU	236
2071	CUUCUUAUA UAUGCUGU	237
2073	UCUUAUAUA UGCUGUUU	238
2080	UAUGCUGUU UCGUUUAU	239
2081	AUGCUGUUU CGUUUAUG	240
2082	UGCUGUUUC GUUUAUGU	241
2085	UGUUUCGUU UAUGUGAU	242
2086	GUUUCGUUU AUGUGAUA	243
2087	UUUCGUUUA UGUGAUAU	244
2094	UAUGUGAUA UGGACAAG	245
2104	GGACAAGUA UGUGUAGC	246
2110	GUAUGUGUA GCUGCUUG	247
2117	UAGCUGCUU GCUUGUGC	248
2121	UGCUUGCUU GUGCUAGU	249
2127	CUUGUGCUA GUGUAAUA	250
2132	GCUAGUGUA AUAUAGUG	251
2135	AGUGUAAUA UAGUGUAG	252
2137	UGUAAUAUA GUGUAGUG	253
2142	UAUAGUGUA GUGGUGGC	254
2165	CACAACCUA AUAAGCGC	255
2168	AACCUAAUA AGCGCAUG	256
2181	CAUGAACUA AUUGCUUG	257
2184	GAACUAAUU GCUUGCGU	258
2188	UAAUUGCUU GCGUGUGU	259
2197	GCGUGUGUA GUUAAGUA	260
2200	UGUGUAGUU AAGUACCG	261
2201	GUGUAGUUA AGUACCGA	262
2205	AGUUAAGUA CCGAUCGG	263
2211	GUACCGAUC GGUAAUUU	264
2215	CGAUCGGUA AUUUUAUA	265
2218	UCGGUAAUU UUAUAUUG	266

nt. Position	Substrate	Seq. ID No.
2219	CGGUAAUUU UAUAUUGC	211
2220	GGUAAUUUU AUAUUGCG	213
2221	GUAAUUUUA UAUUGCGA	215
2223	AAUUUUAUA UUGCGAGU	217
2225	UUUUAUAUU GCGAGUAA	219
2232	UUGCGAGUA AAUAAAUG	221
2236	GAGUAAAUA AAUGGACC	223
2248	GGACCUGUA GUGGUGGA	225

Table III B: Hammerhead Ribozyme Sequence Targeted Against GBSS mRNA

nt. Position	HH Ribozyme Sequence	Seq. ID No.
12	UGGCUGUGGC CUGAUGA X GAA AUCGAUCGGU	267
68	GCAGUGAGUU CUGAUGA X GAA AUUCCUUCCU	268
73	GGCUGGCAGU CUGAUGA X GAA AGUUUAUUCC	269
103	GACGGAGCAG CUGAUGA X GAA ACACUUCUCC	270
109	CUGGUGGACG CUGAUGA X GAA AGCAGUACAC	271
113	CGCACUGGUG CUGAUGA X GAA ACGGAGCAGU	272
146	UCGACGAGAU CUGAUGA X GAA AGCAGCCCUG	273
149	UCGUCGACGA CUGAUGA X GAA AUGAGCAGCC	274
151	GGUCGUCGAC CUGAUGA X GAA AGAUGAGCAG	275
154	ACUGGUCGUC CUGAUGA X GAA ACGAGAUGAG	276
169	CAUGCCGAUU CUGAUGA X GAA AUCCACUGGU	277
170	CCAUGCCGAU CUGAUGA X GAA AAUCCACUGG	278
173	CCGCCAUGCC CUGAUGA X GAA AUUAAUCCAC	279
186	GACGUGGCUA CUGAUGA X GAA AGCCGCCAUG	280
188	GCGACGUGGC CUGAUGA X GAA AGAGCCGCCA	281
196	GACGAGCUGC CUGAUGA X GAA ACGUGGCUAG	282
203	GCGUUGCGAC CUGAUGA X GAA AGCUGCGACG	283
206	CGCGCGUUGC CUGAUGA X GAA ACGAGCUGCG	284
230	ACGCGUCCGG CUGAUGA X GAA ACGCCCAGGC	285
241	GCGGAACGUG CUGAUGA X GAA ACGCGUCCGG	286
247	GCCGCGGCGG CUGAUGA X GAA ACGUGGACGC	287
248	CGCCGCGGCG CUGAUGA X GAA AACGUGGACG	288
292	GUCCGCCGCC CUGAUGA X GAA ACGCCGUCCG	289
308	UCCGAAUGCU CUGAUGA X GAA AGCGUGUCCG	290
314	CGCUGGUCCG CUGAUGA X GAA AUGCUGAGCG	291
315	GCGCUGGUCC CUGAUGA X GAA AAUGCUGAGC	292
344	GCUGGUGCUG CUGAUGA X GAA AGCCUGGGCG	293
385	GAGCGACGGG CUGAUGA X GAA ACCUGGCCCC	294
386	CGAGCGACGG CUGAUGA X GAA AACCUGGCCC	295
391	CACGACGAGC CUGAUGA X GAA ACGGGAACCU	296
395	CGCACACGAC CUGAUGA X GAA AGCGACGGGA	297
398	UGGCGCACAC CUGAUGA X GAA ACGAGCGACG	298
425	CGACGAAGAC CUGAUGA X GAA ACGUUCAUGC	299
428	CGCCGACGAA CUGAUGA X GAA ACGACGUUCA	300
430	GGCGCCGACG CUGAUGA X GAA AGACGACGUU	301
431	CGGCGCCGAC CUGAUGA X GAA AAGACGACGU	302
434	UCUCGGCGCC CUGAUGA X GAA ACGAAGACGA	303
473	GGACGUCGCC CUGAUGA X GAA AGGCCGCCGG	304
482	GGCCGCCGAG CUGAUGA X GAA ACGUCGCCGA	305
485	GCAGGCCGCC CUGAUGA X GAA AGGACGUCGC	306
527	AGACGACCAU CUGAUGA X GAA ACACGGUGCC	307
533	GGGGAGAGAC CUGAUGA X GAA ACCAUGACAC	308
536	AGCGGGGAGA CUGAUGA X GAA ACGACCAUGA	309
538	GUAGCGGGGA CUGAUGA X GAA AGACGACCAU	310
540	UCGUAGCGGG CUGAUGA X GAA AGAGACGACC	311

Table IIIB

nt. Positi n	HH Ribozyme Sequence	Seq. ID No.
547	GUACUGGUCG CUGAUGA X GAA AGCGGGGAGA	312
556	GGCGUCCUUG CUGAUGA X GAA ACUGGUCGUA	313
581	UCUCGGACAC CUGAUGA X GAA ACGCUGGUGU	314
586	CUUGAUCUCG CUGAUGA X GAA ACACGACGCU	315
593	CUCCCAUCUU CUGAUGA X GAA AUCUCGGACA	316
610	GACCGUCUCG CUGAUGA X GAA ACCUGUCUCC	317
620	GGAAGAACCU CUGAUGA X GAA ACCGUCUCGU	318
625	GCAGUGGAAG CUGAUGA X GAA ACCUGACCGU	319
626	AGCAGUGGAA CUGAUGA X GAA AACCUGACCG	320
628	GUAGCAGUGG CUGAUGA X GAA AGAACCUGAC	321
629	UGUAGCAGUG CUGAUGA X GAA AAGAACCUGA	322
637	UCCGCGCUUG CUGAUGA X GAA AGCAGUGGAA	323
661	GUGGUCAACG CUGAUGA X GAA ACACGCGGUC	324
662	GGUGGUCAAC CUGAUGA X GAA AACACGCGGU	325
665	GUGGGUGGUC CUGAUGA X GAA ACGAACACGC	326
679	CCUCUCCAGG CUGAUGA X GAA ACAGUGGGUG	327
680	CCCUCUCCAG CUGAUGA X GAA AACAGUGGGU	328
692	UCUUUCCCCA CUGAUGA X GAA ACCCUCUCCA	329
693	GUCUUUCCCC CUGAUGA X GAA AACCCUCUCC	330
716	CAGGCCCGUA CUGAUGA X GAA AUCUUCUCCU	331
718	GUCAGGCCCG CUGAUGA X GAA AGAUCUUCUC	332
742	GUUGUCCCUG CUGAUGA X GAA AGUCCGUUCC	333
763	UAGCAGGCUG CUGAUGA X GAA ACCGCAGCUG	334
764	AUAGCAGGCU CUGAUGA X GAA AACCGCAGCU	335
773	CUGCCUGGCA CUGAUGA X GAA AGCAGGCUGA	336
788	UUGGAGCUUC CUGAUGA X GAA AGUGCUGCCU	337
795	AGGAUCCUUG CUGAUGA X GAA AGCUUCAAGU	338
803	UGAGGCUCAG CUGAUGA X GAA AUCCUUGGAG	339
812	GGUUGUUGUU CUGAUGA X GAA AGGCUCAGGA	340
826	UCCGGAGAAG CUGAUGA X GAA AUGGGUUGUU	341
829	UGGUCCGGAG CUGAUGA X GAA AGUAUGGGUU	342
830	AUGGUCCGGA CUGAUGA X GAA AAGUAUGGGU	343
832	GUAUGGUCCG CUGAUGA X GAA AGAAGUAUGG	344
841	GUCCUCCCCG CUGAUGA X GAA AUGGUCCGGA	345
854	AGACGAACAC CUGAUGA X GAA ACGUCCUCCC	346
859	GUUGCAGACG CUGAUGA X GAA ACACGACGUC	347
860	CGUUGCAGAC CUGAUGA X GAA AACACGACGU	348
863	AGUCGUUGCA CUGAUGA X GAA ACGAACACGA	349
888	UAGCACGAGA CUGAUGA X GAA AGGGCCGGUG	350
890	GGUAGCACGA CUGAUGA X GAA AGAGGGCCGG	351
892	GAGGUAGCAC CUGAUGA X GAA AGAGAGGGCC	352
898	GCUCUUGAGG CUGAUGA X GAA AGCACGAGAG	353
902	AGUUGCUCUU CUGAUGA X GAA AGGUAGCACG	354
913	GUGGGACUGG CUGAUGA X GAA AGUUGCUCUU	355
919	GAUGCCGUGG CUGAUGA X GAA ACUGGUAGUU	356
929	CGUCCCUGUA CUGAUGA X GAA AUGCCGUGGG	357
931	UGCGUCCCUG CUGAUGA X GAA AGAUGCCGUG	358
951	UGGAUGCAGA CUGAUGA X GAA AGCGGUCUUU	359
952	GUGGAUGCAG CUGAUGA X GAA AAGCGGUCUU	360
953	UGUGGAUGCA CUGAUGA X GAA AAAGCGGUCU	361
959	AGAUGUUGUG CUGAUGA X GAA AUGCAGAAAG	362
968	CCUGGUAGGA CUGAUGA X GAA AUGUUGUGGA	363

Table IIIB

nt. Position	HH Rib zyme Sequence	Seq. ID No.
970	GCCCUGGUAG CUGAUGA X GAA AGAUGUUGUG	364
973	CCGGCCCUGG CUGAUGA X GAA AGGAGAUGUU	365
985	GGAGAAGGCG CUGAUGA X GAA ACCGGCCCUG	366
986	CGGAGAAGGC CUGAUGA X GAA AACCGGCCCU	367
991	GUAGUCGGAG CUGAUGA X GAA AGGCGAACCG	368
992	GGUAGUCGGA CUGAUGA X GAA AAGGCGAACC	369
994	CGGGUAGUCG CUGAUGA X GAA AGAAGGCGAA	370
1000	CAGCUCCGGG CUGAUGA X GAA AGUCGGAGAA	371
1016	AUCUCUCCGG CUGAUGA X GAA AGGUUCAGCU	372
1027	GGACGACUUG CUGAUGA X GAA AUCUCUCCGG	373
1028	AGGACGACUU CUGAUGA X GAA AAUCUCUCCG	374
1033	AUCGAAGGAC CUGAUGA X GAA ACUUGAAUCU	375
1036	GAAAUCGAAG CUGAUGA X GAA ACGACUUGAA	376
1039	GAUGAAAUCG CUGAUGA X GAA AGGACGACUU	377
1040	CGAUGAAAUC CUGAUGA X GAA AAGGACGACU	378
1044	CCGUCGAUGA CUGAUGA X GAA AUCGAAGGAC	379
1045	GCCGUCGAUG CUGAUGA X GAA AAUCGAAGGA	380
1046	AGCCGUCGAU CUGAUGA X GAA AAAUCGAAGG	381
1049	CGUAGCCGUC CUGAUGA X GAA AUGAAAUCGA	382
1057	GGGCUUCUCG CUGAUGA X GAA AGCCGUCGAU	383
1085	UCAUCCAGUU CUGAUGA X GAA AUCUUCCGGC	384
1106	CGGCCUCGAG CUGAUGA X GAA AUCCCGGCCU	385
1109	UGUCGGCCUC CUGAUGA X GAA AGGAUCCCGG	386
1124	UGACGGUGAG CUGAUGA X GAA ACCCUGUCGG	387
1127	GGCUGACGGU CUGAUGA X GAA AGGACCCUGU	388
1133	AGUAGGGGCU CUGAUGA X GAA ACGGUGAGGA	389
1141	CUCGGCGUAG CUGAUGA X GAA AGGGGCUGAC	390
1144	CUCCUCGGCG CUGAUGA X GAA AGUAGGGGCU	391
1157	UGCCGGAGAU CUGAUGA X GAA AGCUCCUCGG	392
1160	CGAUGCCGGA CUGAUGA X GAA AUGAGCUCCU	393
1162	GGCGAUGCCG CUGAUGA X GAA AGAUGAGCUC	394
1169	AGCCCCUGGC CUGAUGA X GAA AUGCCGGAGA	395
1187	UGAUGUUGUC CUGAUGA X GAA AGCUCGCAGC	396
1196	UGAGGCGCAU CUGAUGA X GAA AUGUUGUCGA	397
1205	UGAUGCCGGU CUGAUGA X GAA AGGCGCAUGA	398
1214	CGAUGCCGGU CUGAUGA X GAA AUGCCGGUGA	399
1223	UGCCGUUGAC CUGAUGA X GAA AUGCCGGUGA	400
1226	CCAUGCCGUU CUGAUGA X GAA ACGAUGCCGG	401
1241	CCCACUCGCU CUGAUGA X GAA ACGUCCAUGC	402
1270	CACGGCGAUG CUGAUGA X GAA ACUUGUCCCU	403
1274	ACUUCACGGC CUGAUGA X GAA AUGUACUUGU	404
1285	CGACACGUCG CUGAUGA X GAA ACUUCACGGC	405
1294	CACGGCCGUC CUGAUGA X GAA ACACGUCGUA	406
1346	CCGGGAGCCC CUGAUGA X GAA ACCUCCGCCU	407
1352	GGUCCACCGG CUGAUGA X GAA AGCCCGACCU	408
1370	CCACCAGCGG CUGAUGA X GAA AUGUUCCGGU	409
1384	CCUGCCGAUG CUGAUGA X GAA ACGCCACCAG	410
1385	GCCUGCCGAU CUGAUGA X GAA AACGCCACCA	411
1388	CCAGCCUGCC CUGAUGA X GAA AUGAACGCCA	412
1421	CGGCCGCCAU CUGAUGA X GAA ACGUCGGGUC	413
1436	UGAGCUGCGG CUGAUGA X GAA AUGGCGGCCG	414
1445	CCAUCUCCAU CUGAUGA X GAA AGCUGCGGGA	415

Table IIIB

nt. P siti n	HH Rib zyme Sequence	Seq. ID No.
1472	CCAGCAGAAC CUGAUGA X GAA AUCUGCACGU	416
1475	UGCCCAGCAG CUGAUGA X GAA ACGAUCUGCA	417
1476	GUGCCCAGCA CUGAUGA X GAA AACGAUCUGC	418
1501	CAUGCGCUCG CUGAUGA X GAA ACUUCUUCUU	419
1502	GCAUGCGCUC CUGAUGA X GAA AACUUCUUCU	420
1514	CGGCGCUCAU CUGAUGA X GAA AGCAUGCGCU	421
1534	CUUGCCUGGG CUGAUGA X GAA ACUUCUCCUC	422
1535	CCUUGCCUGG CUGAUGA X GAA AACUUCUCCU	423
1559	CGUUGAACUU CUGAUGA X GAA ACCACGGCGC	424
1564	CGCCGCGUUG CUGAUGA X GAA ACUUGACCAC	425
1565	GCGCCGCGUU CUGAUGA X GAA AACUUGACCA	426
1589	CGCCGGCCAU CUGAUGA X GAA AUGUGGUGCG	427
1610	UGGUGACGGC CUGAUGA X GAA AGCACGUCGG	428
1616	AGCGGCUGGU CUGAUGA X GAA ACGGCGAGCA	429
1627	GCAGGGCUCG CUGAUGA X GAA AGCGGCUGGU	430
1628	CGCAGGGCUC CUGAUGA X GAA AAGCGGCUGG	431
1643	GCAGCUGGAU CUGAUGA X GAA AGGCCGCAGG	432
1646	CCUGCAGCUG CUGAUGA X GAA AUGAGGCCGC433	
1666	GGGCGUUCCG CUGAUGA X GAA AUCGCAUCCC	434
1690	UCCACCGGUG CUGAUGA X GAA ACGCGCAGGC	435
1703	UGGUGUCGAC CUGAUGA X GAA AGUCCACCGG	436
1706	UGAUGGUGUC CUGAUGA X GAA ACGAGUCCAC	437
1715	UGCCUUCGAU CUGAUGA X GAA AUGGUGUCGA	438
1718	UCUUGCCUUC CUGAUGA X GAA AUGAUGGUGU	439
1735	GCCCAUGUGG CUGAUGA X GAA ACCCGGUCUU	440
1736	GGCCCAUGUG CUGAUGA X GAA AACCCGGUCU	441
1751	AGUCGACGCU CUGAUGA X GAA AGGCGGCCCA	442
1757	CGUUGCAGUC CUGAUGA X GAA ACGCUGAGGC	443
1769	CCGGCUCCAC CUGAUGA X GAA ACGUUGCAGU	444
1787	CCACCUUCUU CUGAUGA X GAA ACGUCCGCCG	445
1807	GGCGCGCUGC CUGAUGA X GAA AGGUGGUGGC	446
1820	CGACCACCUU CUGAUGA X GAA AUGGCGCGCU	447
1829	CCGGCGUGCC CUGAUGA X GAA ACCACCUUGA	448
1843	CAUCUCCUCG CUGAUGA X GAA ACGCCGGCGU	449
1871	AGAGAUCCUG CUGAUGA X GAA AUCAUGCAGU	450
1878	UUCCAGGAGA CUGAUGA X GAA AUCCUGGAUC	451
1880	CCUUCCAGGA CUGAUGA X GAA AGAUCCUGGA	452
1882	GCCCUUCCAG CUGAUGA X GAA AGAGAUCCUG	453
1922	CCCCGAGGCU CUGAUGA X GAA AGCAGCACGU	454
1928	CGGCGACCCC CUGAUGA X GAA AGGCUGAGCA	455
1934	CGCCGCCGGC CUGAUGA X GAA ACCCCGAGGC	456
1955	CCUCGCCUUC CUGAUGA X GAA ACCCCUGGCU	457
1970	CGAGCGGCGC CUGAUGA X GAA AUCUCCUCGC	458
1979	UCUCCUUGGC CUGAUGA X GAA AGCGGCGCGA	459
2012	CUGCAGGCCG CUGAUGA X GAA ACUCUUCAGG	460
2013	CCUGCAGGCC CUGAUGA X GAA AACUCUUCAG	461
2033	CCACGCGCGA CUGAUGA X GAA AUCAGGGGGC	462
2035	CACCACGCGC CUGAUGA X GAA AGAUCAGGGG	463
2055	AAGAUGUCCC CUGAUGA X GAA ACAUGUUUGC	464
2063	UAUAUAAGAA CUGAUGA X GAA AUGUCCCAAC	465
2065	CAUAUAUAAG CUGAUGA X GAA AGAUGUCCCA	466
2066	GCAUAUAUAA CUGAUGA X GAA AAGAUGUCCC	467

Table IIIB

nt. Position	HH Ribozyme Sequence	Seq. ID No.
2068	CAGCAUAUAU CUGAUGA X GAA AGAAGAUGUC	468
2069	ACAGCAUAUA CUGAUGA X GAA AAGAAGAUGU	469
2071	AAACAGCAUA CUGAUGA X GAA AUAAGAAGAU	470
2073	CGAAACAGCA CUGAUGA X GAA AUAUAAGAAG	471
2080	ACAUAAACGA CUGAUGA X GAA ACAGCAUAUA	472
2081	CACAUAAACG CUGAUGA X GAA AACAGCAUAU	473
2082	UCACAUAAAC CUGAUGA X GAA AAACAGCAUA	474
2085	AUAUCACAUA CUGAUGA X GAA ACGAAACAGC	475
2086	CAUAUCACAU CUGAUGA X GAA AACGAAACAG	476
2087	CCAUAUCACA CUGAUGA X GAA AAACGAAACA	477
2094	UACUUGUCCA CUGAUGA X GAA AUCACAUAAA	478
2104	CAGCUACACA CUGAUGA X GAA ACUUGUCCAU	479
2110	AGCAAGCAGC CUGAUGA X GAA ACACAUACUU	480
2117	UAGCACAAGC CUGAUGA X GAA AGCAGCUACA	481
2121	ACACUAGCAC CUGAUGA X GAA AGCAAGCAGC	482
2127	UAUAUUACAC CUGAUGA X GAA AGCACAAGCA	483
2132	UACACUAUAU CUGAUGA X GAA ACACUAGCAC	484
2135	CACUACACUA CUGAUGA X GAA AUUACACUAG	485
2137	ACCACUACAC CUGAUGA X GAA AUAUUACACU	486
2142	UGGCCACCAC CUGAUGA X GAA ACACUAUAUU	487
2165	AUGCGCUUAU CUGAUGA X GAA AGGUUGUGCC	488
2168	UUCAUGCGCU CUGAUGA X GAA AUUAGGUUGU	489
2181	CGCAAGCAAU CUGAUGA X GAA AGUUCAUGCG	490
2184	ACACGCAAGC CUGAUGA X GAA AUUAGUUCAU	491
2188	CUACACACGC CUGAUGA X GAA AGCAAUUAGU	492
2197	GGUACUUAAC CUGAUGA X GAA ACACACGCAA	493
2200	AUCGGUACUU CUGAUGA X GAA ACUACACACG	494
2201	GAUCGGUACU CUGAUGA X GAA AACUACACAC	495
2205	UACCGAUCGG CUGAUGA X GAA ACUUAACUAC	496
2211	UAAAAUUACC CUGAUGA X GAA AUCGGUACUU	497
2215	AAUAUAAAAU CUGAUGA X GAA ACCGAUCGGU	498
2218	CGCAAUAUAA CUGAUGA X GAA AUUACCGAUC	499
2219	UCGCAAUAUA CUGAUGA X GAA AAUUACCGAU	500
2220	CUCGCAAUAU CUGAUGA X GAA AAAUUACCGA	501
2221	ACUCGCAAUA CUGAUGA X GAA AAAAUUACCG	502
2223	UUACUCGCAA CUGAUGA X GAA AUAAAAUUAC	503
2225	AUUUACUCGC CUGAUGA X GAA AUAUAAAAUU	504
2232	UCCAUUUAUU CUGAUGA X GAA ACUCGCAAUA	505
2236	CAGGUCCAUU CUGAUGA X GAA AUUUACUCGC	506
2248	UUUCCACCAC CUGAUGA X GAA ACAGGUCCAU	507

Where "X" represents stem II region of a HH ribozyme (Hertel et al., 1992 *Nucleic Acids Res.* 20 3252). The length of stem II may be ≥ 2 base-pairs.

Table IV

Table IV: HH Ribozyme Sequences Tested against GBSS mRNA

nt. Position	HH Ribozyme Sequence	Sequence I.D.
425	CGACGAAGAC CUGAUGAGGCCGAAAGGCCGAA ACGUUCAUGC	2
593	CUCCCAUCUU CUGAUGAGGCCGAAAGGCCGAA AUCUCGGACA	3
742	GUUGUCCCUG CUGAUGAGGCCGAAAGGCCGAA AGUCCGUUCC	4
812	GGUUGUUGUU CUGAUGAGGCCGAAAGGCCGAA AGGCUCAGGA	5
892	GAGGUAGCAC CUGAUGAGGCCGAAAGGCCGAA AGAGAGGGCC	6
913	GUGGGACUGG CUGAUGAGGCCGAAAGGCCGAA AGUUGCUCUU	7
919	GAUGCCGUGG CUGAUGAGGCCGAAAGGCCGAA ACUGGUAGUU	8
953	UGUGGAUGCA CUGAUGAGGCCGAAAGGCCGAA AAAGCGGUCU	9
959	AGAUGUUGUG CUGAUGAGGCCGAAAGGCCGAA AUGCAGAAAG	10
968	CCUGGUAGGA CUGAUGAGGCCGAAAGGCCGAA AUGUUGUGGA	11
1016	AUCUCUCCGG CUGAUGAGGCCGAAAGGCCGAA AGGUUCAGCU	12
1028	AGGACGACUU CUGAUGAGGCCGAAAGGCCGAA AAUCUCUCCG	13
1085	UCAUCCAGUU CUGAUGAGGCCGAAAGGCCGAA AUCUUCCGGC	14
1187	UGAUGUUGUC CUGAUGAGGCCGAAAGGCCGAA AGCUCGCAGC	15
1196	UGAGGCGCAU CUGAUGAGGCCGAAAGGCCGAA AUGUUGUCGA	16
1226	CCAUGCCGUU CUGAUGAGGCCGAAAGGCCGAA ACGAUGCCGG	17
1241	CCCACUCGCU CUGAUGAGGCCGAAAGGCCGAA ACGUCCAUGC	18
1270	CACGGCGAUG CUGAUGAGGCCGAAAGGCCGAA ACUUGUCCCU	19
1352	GGUCCACCGG CUGAUGAGGCCGAAAGGCCGAA AGCCCGACCU	20
1421	CGGCCGCCAU CUGAUGAGGCCGAAAGGCCGAA ACGUCGGGUC	21
1534	CUUGCCUGGG CUGAUGAGGCCGAAAGGCCGAA ACUUCUCCUC	22
1715	UGCCUUCGAU CUGAUGAGGCCGAAAGGCCGAA AUGGUGUCGA	23
1787	CCACCUUCUU CUGAUGAGGCCGAAAGGCCGAA ACGUCCGCCG	24

Table VA

Table V A: GBSS Hairpin Ribozyme and Substrate Sequences

nt. Position	Hairpin Ribozyme Sequence	Seq. ID No.	Substrate	Seq. ID No.
48	CUCCUGGC AGAA GUCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	508	CGACA GCC GCCAGGAG	509
129	CCCUGCCG AGAA GUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	510	GCACC GCC CGGCAGGG	511
468	GUCGCCGA AGAA GCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	512	CGGCG GCC UCGGCGAC	513
489	CGGCGGCA AGAA GCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	514	CGGCG GCC UGCCGCCG	515
496	CCAUGGCC AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	516	CUGCC GCC GGCCAUGG	517
676	UCUCCAGG AGAA GUGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	518	CCACU GUU CCUGGAGA	519
737	UCCCUGUA AGAA GUUC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	520	GAACG GAC UACAGGGA	521
760	GCAGGCUG AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	522	CUGCG GUU CAGCCUGC	523
1298	GCCUCCAC AGAA GUCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	524	CGACG GCC GUGGAGGC	525
1427	GGGAUGGC AGAA GCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	526	UGGCG GCC GCCAUCCC	527
1601	GCGAGCAC AGAA GCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	528	GCGCC GAC GUGCUCGC	529
1638	CUGGAUGA AGAA GCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	530	CUGCG GCC UCAUCCAG	531
1746	GACGCUGA AGAA GCCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	532	GGGCC GCC UCAGCGUC	533
1781	UUCUUGAC AGAA GCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	534	CGGCG GAC GUCAAGAA	535
2077	AUAAACGA AGAA GCAU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	536	AUGCU GUU UCGUUUAU	537

Table VB

Table VB: GBSS Hairpin Ribozyme and Substrate Sequences

t. Position	Ribozyme Sequence	Seq. ID No.	Substrate	Seq. ID No.
31	GUCGCCUC AGAA GGUGGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	538	ACCACCC GCC GAGGCGAC	539
48	CUCCUGGC AGAA GUCGCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	540	CGCGACA GCC GCCAGGAG	541
105	GUGGACGG AGAA GUACAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	542	GUGUACU GCU CCGUCCAC	543
110	CACUGGUG AGAA GAGCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	544	CUGCUCC GUC CACCAGUG	545
129	CCCUGCCG AGAA GUGCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	546	GCGCACC GCC CGGCAGGG	547
142	ACGAGAUG AGAA GCCCUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	548	CAGGGCU GCU CAUCUCGU	549
182	GUGGCUAG AGAA GCCAUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	550	CAUGGCG GCU CUAGCCAC	551
199	UUGCGACG AGAA GCGACG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	552	CGUCGCA GCU CGUCGCAA	553
219	GACGCCCA AGAA GGCGCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	554	CGCGCCG GCC UGGGCGUC	555
233	GUGGACGC AGAA GGGACG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	556	CGUCCCG GAC GCGUCCAC	557
249	GGCGCCGC AGAA GAACGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	558	ACGUUCC GCC GCGGCGCC	559
283	CCGACGCC AGAA GGCCCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	560	GGGGCCG GAC GGCGUCGG	561
316	GCGCGCUG AGAA GAAUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	562	GCAUUCG GAC CAGCGCGC	563
388	CGACGAGC AGAA GGAACC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	564	GGUUCCC GUC GCUCGUCG	565
468	GUCGCCGA AGAA GCCGGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	566	ACCGGCG GCC UCGGCGAC	567
489	CGGCGGCA AGAA GCCGAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	568	CUCGGCG GCC UGCCGCCG	569
493	UGGCCGGC AGAA GGCCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	570	GCGGCCU GCC GCCGGCCA	571
496	CCAUGGCC AGAA GCAGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	572	GCCUGCC GCC GGCCAUGG	573
676	UCUCCAGG AGAA GUGGGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	574	ACCCACU GUU CCUGGAGA	575
725	GUUCCAGC AGAA GGCCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	576	CGGGCCU GAC GCUGGAAC	577
737	UCCCUGUA AGAA GUUCCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	578	UGGAACG GAC UACAGGGA	579
754	UGAACCGC AGAA GGUUGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	580	ACAACCA GCU GCGGUUCA	581
760	GCAGGCUG AGAA GCAGCU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	582	AGCUGCG GUU CAGCCUGC	583
765	GCAUAGCA AGAA GAACCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	584	CGGUUCA GCC UGCUAUGC	585
834	CCCGUAUG AGAA GGAGAA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	586	UUCUCCG GAC CAUACGGG	587
882	CGAGAGAG AGAA GGUGUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	588	CACACCG GCC CUCUCUCG	589
916	UGCCGUGG AGAA GGUAGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	590	ACUACCA GUC CCACGGCA	591
947	AUGCAGAA AGAA GUCUUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	592	AAAGACC GCU UUCUGCAU	593
982	AGAAGGCG AGAA GGCCCU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	594	AGGGCCG GUU CGCCUUCU	595
995	UCCGGGUA AGAA GAGAAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	596	CUUCUCC GAC UACCCGGA	597
1134	GUAGUAGG AGAA GACGGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	598	ACCGUCA GCC CCUACUAC	599
1298	GCCUCCAC AGAA GUCGAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	600	GUCGACG GCC GUGGAGGC	601

Table VB

. P sition	Ribozyme Sequence	Seq. ID No.	Substrate	Seq. ID No.
1372	ACGCCACC AGAA GGAUGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	602	ACAUCCC GCU GGUGGCGU	603
1415	GCCAUGAC AGAA GGUCCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	604	GGGACCC GAC GUCAUGGC	605
1427	GGGAUGGC AGAA GCCAUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	606	CAUGGCG GCC GCCAUCCC	607
1441	UCUCCAUG AGAA GCGGGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	608	UCCCGCA GCU CAUGGAGA	609
1468	GCAGAACG AGAA GCACGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	610	ACGUGCA GAU CGUUCUGC	611
1477	CCGUGCCC AGAA GAACGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	612	UCGUUCU GCU GGGCACGG	613
1601	GCGAGCAC AGAA GCGCCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	614	CGGCGCC GAC GUGCUCGC	615
1620	CUCGAAGC AGAA GGUGAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	616	GUCACCA GCC GCUUCGAG	617
1623	GGGCUCGA AGAA GCUGGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	618	ACCAGCC GCU UCGAGCCC	619
1638	CUGGAUGA AGAA GCAGGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	620	CCCUGCG GCC UCAUCCAG	621
1648	UCCCCUGC AGAA GGAUGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	622	UCAUCCA GCU GCAGGGGA	623
1746	GACGCUGA AGAA GCCCAU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	624	AUGGGCC GCC UCAGCGUC	625
1781	UUCUUGAC AGAA GCCGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	626	GCCGGCG GAC GUCAAGAA	627
1918	CGAGGCUG AGAA GCACGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	628	ACGUGCU GCU CAGCCUCG	629
1923	GACCCCGA AGAA GAGCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	630	CUGCUCA GCC UCGGGGUC	631
1975	CCUUGGCG AGAA GCGCGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	632	UCGCGCC GCU CGCCAAGG	633
2014	GGCCUGCA AGAA GAACUC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	634	GAGUUCG GCC UGCAGGCC	635
2029	CGCGCGAG AGAA GGGGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	636	GCCCCCU GAU CUCGCGCG	637
2077	AUAAACGA AGAA GCAUAU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	638	AUAUGCU GUU UCGUUUAU	639
2113	CACAAGCA AGAA GCUACA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	640	UGUAGCU GCU UGCUUGUG	641
2207	AAUUACCG AGAA GUACUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	642	AAGUACC GAU CGGUAAUU	643

Table VI

Table VI: Delta-9 Desaturase HH Ribozyme Target Sequences

nt. Position	Substrate	Seq. ID No.	nt. Position	Substrate	Seq. ID N .
13	CGCGCCCUC UGCCGCUU	644	319	GUCCAGGUU ACACAUUC	645
21	CUGCCGCUU GUUCGUUC	646	320	UCCAGGUUA CACAUUCA	647
24	CCGCUUGUU CGUUCCUC	648	326	UUACACAUU CAAUGCCA	649
25	CGCUUGUUC GUUCCUCG	650	327	UACACAUUC AAUGCCAC	651
28	UUGUUCGUU CCUCGCGC	652	338	UGCCACCUC ACAAGAUU	653
29	UGUUCGUUC CUCGCGCU	654	346	CACAAGAUU GAAAUUUU	655
32	UCGUUCCUC GCGCUCGC	656	352	AUUGAAAUU UUCAAGUC	657
38	CUCGCGCUC GCCACCAG	658	353	UUGAAAUUU UCAAGUCG	659
63	ACACACAUC CCAAUCUC	660	354	UGAAAUUUU CAAGUCGC	661
69	AUCCCAAUC UCGCGAGG	662	355	GAAAUUUUC AAGUCGCU	663
71	CCCAAUCUC GCGAGGGC	664	360	UUUCAAGUC GCUUGAUG	665
92	AGCAGGGUC UGCGGCGG	666	364	AAGUCGCUU GAUGAUUG	667
117	GCCGCGCUU CCGGCUCC	668	371	UUGAUGAUU GGGCUAGA	669
118	CCGCGCUUC CGGCUCCC	670	377	AUUGGGCUA GAGAUAAU	671
124	UUCCGGCUC CCCUUCCC	672	383	CUAGAGAUA AUAUCUUG	673
129	GCUCCCCUU CCCAUUGG	674	386	GAGAUAAUA UCUUGACG	675
130	CUCCCCUUC CCAUUGGC	676	388	GAUAAUAUC UUGACGCA	677
135	CUUCCCAUU GGCCUCCA	678	390	UAAUAUCUU GACGCAUC	679
141	AUUGGCCUC CACGAUGG	680	398	UGACGCAUC UCAAGCCA	681
154	AUGGCGCUC CGCCUCAA	682	400	ACGCAUCUC AAGCCAGU	683
160	CUCCGCCUC AACGACGU	684	409	AAGCCAGUC GAGAAGUG	685
169	AACGACGUC GCGCUCUG	686	419	AGAAGUGUU GGCAGCCA	687
175	GUCGCGCUC UGCCCUCUC	688	434	CACAGGAUU UCCUCCCG	689
181	CUCUGCCUC UCCCCGCC	690	435	ACAGGAUUU CCUCCCGG	691
183	CUGCCUCUC CCCGCCGC	692	436	CAGGAUUUC CUCCCGGA	693
193	CCGCCGCUC GCCGCCCG	694	439	GAUUUCCUC CCGGACCC	695
228	CGGCAGGUU CGUCGCCG	696	453	CCCAGCAUC UGAAGGAU	697
229	GGCAGGUUC GUCGCCGU	698	462	UGAAGGAUU UCAUGAUG	699
232	AGGUUCGUC GCCGUCGC	700	463	GAAGGAUUU CAUGAUGA	701
238	GUCGCCGUC GCCUCCAU	702	464	AAGGAUUUC AUGAUGAA	703
243	CGUCGCCUC CAUGACGU	704	475	GAUGAAGUU AAGGAGCU	705
252	CAUGACGUC CGCCGUCU	706	476	AUGAAGUUA AGGAGCUC	707
259	UCCGCCGUC UCCACCAA	708	484	AAGGAGCUC AGAGAACG	709
261	CGCCGUCUC CACCAAGG	710	505	AAGGAAAUC CCUGAUGA	711
271	ACCAAGGUC GAGAAUAA	712	515	CUGAUGAUU AUUUUGUU	713
278	UCGAGAAUA AGAAGCCA	714	516	UGAUGAUUA UUUUGUUU	715
288	GAAGCCAUU UGCUCCUC	716	518	AUGAUUAUU UUGUUUGU	717
289	AAGCCAUUU GCUCCUCC	718	519	UGAUUAUUU UGUUUGUU	719
293	CAUUUGCUC CUCCAAGG	720	520	GAUUAUUUU GUUUGUUU	721
296	UUGCUCCUC CAAGGGAG	722	523	UAUUUUGUU UGUUUGGU	723
307	AGGGAGGUA CAUGUCCA	724	524	AUUUUGUUU GUUUGGUG	725
313	GUACAUGUC CAGGUUAC	726	527	UUGUUUGUU UGGUGGGA	727
528	UGUUUGUUU GGUGGGAG	728	857	ACACUGCUC GUCACGCC	729
544	GACAUGAUU ACCGAGGA	730	860	CUGCUCGUC ACGCCAAG	731
545	ACAUGAUUA CCGAGGAA	732	873	CAAGGACUU UGGCGACU	733
557	AGGAAGCUC UACCAACA	734	874	AAGGACUUU GGCGACUU	735
559	GAAGCUCUA CCAACAUA	736	882	UGGCGACUU AAAGCUUG	737
567	ACCAACAUA CCAGACUA	738	883	GGCGACUUA AAGCUUGC	739
575	ACCAGACUA UGCUUAAC	740	889	UUAAAGCUU GCACAAAU	741
580	ACUAUGCUU AACACCCU	742	898	GCACAAAUC UGCGGCAU	743
581	CUAUGCUUA ACACCCUC	744	907	UGCGGCAUC AUCGCCUC	745
589	AACACCCUC GACGGUGU	746	910	GGCAUCAUC GCCUCAGA	747
598	GACGGUGUC AGAGAUGA	748	915	CAUCGCCUC AGAUGAGA	749

Table VI

nt. Position	Substrate	Seq. ID No.	nt. Position	Substrate	Seq. ID No.
637	UGGGCUGUU UGGACGAG	750	942	AACUGCGUA CACCAAGA	751
638	GGGCUGUUU GGACGAGG	752	952	ACCAAGAUC GUGGAGAA	753
680	AUGGUGAUC UGCUCAAC	754	966	GAAGCUGUU UGAGAUCG	755
685	GAUCUGCUC AACAAGUA	756	967	AAGCUGUUU GAGAUCGA	757
693	CAACAAGUA UAUGUACC	758	973	UUUGAGAUC GACCCUGA	759
695	ACAAGUAUA UGUACCUC	760	986	CUGAUGGUA CCGUGGUC	761
699	GUAUAUGUA CCUCACUG	762	994	ACCGUGGUC GCUCUGGC	763
703	AUGUACCUC ACUGGGAG	764	998	UGGUCGCUC UGGCUGAC	765
719	GGGUGGAUA UGAGGCAG	766	1024	AAGAAGAUC UCAAUGCC	767
730	AGGCAGAUU GAGAAGAC	768	1026	GAAGAUCUC AAUGCCUG	769
742	AAGACAAUU CAGUAUCU	770	1047	CCUGAUGUU UGACGGGC	771
743	AGACAAUUC AGUAUCUU	772	1048	CUGAUGUUU GACGGGCA	773
747	AAUUCAGUA UCUUAUUG	774	1071	CAAGCUGUU CGAGCACU	775
749	UUCAGUAUC UUAUUGGC	776	1072	AAGCUGUUC GAGCACUU	777
751	CAGUAUCUU AUUGGCUC	778	1080	CGAGCACUU CUCCAUGG	779
752	AGUAUCUUA UUGGCUCU	780	1081	GAGCACUUC UCCAUGGU	781
754	UAUCUUAUU GGCUCUGG	782	1083	GCACUUCUC CAUGGUCG	783
759	UAUUGGCUC UGGAAUGG	784	1090	UCCAUGGUC GCGCAGAG	785
770	GAAUGGAUC CUAGGACU	786	1102	CAGAGGCUU GGCGUUUA	787
773	UGGAUCCUA GGACUGAG	788	1108	CUUGGCGUU UACACCGC	789
785	CUGAGAAUA AUCCUUAU	790	1109	UUGGCGUUU ACACCGCC	791
788	AGAAUAAUC CUUAUCUU	792	1110	UGGCGUUUA CACCGCCA	793
791	AUAAUCCUU AUCUUGGU	794	1125	CAGGGACUA CGCCGACA	795
792	UAAUCCUUA UCUUGGUU	796	1135	GCCGACAUC CUCGAGUU	797
794	AUCCUUAUC UUGGUUUC	798	1138	GACAUCCUC GAGUUCCU	799
796	CCUUAUCUU GGUUUCAU	800	1143	CCUCGAGUU CCUCGUCG	801
800	AUCUUGGUU UCAUCUAC	802	1144	CUCGAGUUC CUCGUCGA	803
801	UCUUGGUUU CAUCUACA	804	1147	GAGUUCCUC GUCGACAG	805
802	CUUGGUUUC AUCUACAC	806	1150	UUCCUCGUC GACAGGUG	807
805	GGUUUCAUC UACACCUC	808	1181	UGACUGGUC UGUCGGGU	809
807	UUUCAUCUA CACCUCCU	810	1185	UGGUCUGUC GGGUGAAG	811
813	CUACACCUC CUUCCAAG	812	1212	GCAGGACUA CCUUUGCA	813
816	CACCUCCUU CCAAGAGC	814	1216	GACUACCUU UGCACCCU	815
817	ACCUCCUUC CAAGAGCG	816	1217	ACUACCUUU GCACCCUU	817
834	GGCGACCUU CAUCUCAC	818	1225	UGCACCCUU GCUUCAAG	819
835	GCGACCUUC AUCUCACA	820	1229	CCCUUGCUU CAAGAAUC	821
838	ACCUUCAUC UCACACGG	822	1230	CCUUGCUUC AAGAAUCA	823
840	CUUCAUCUC ACACGGGA	824	1237	UCAAGAAUC AGGAGGCU	825
1292	CGCUGCCUU UCAGCUGG	826	1494	UUUGAUGUA CAACCUGU	827
1293	GCUGCCUUU CAGCUGGG	828	1546	CAUGCCGUA CUUUGUCU	829
1294	CUGCCUUUC AGCUGGGU	830	1549	GCCGUACUU UGUCUGUC	831
1303	AGCUGGGUA UACGGUAG	832	1550	CCGUACUUU GUCUGUCG	833
1305	CUGGGUAUA CGGUAGGG	834	1553	UACUUUGUC UGUCGCUG	835
1310	UAUACGGUA GGGACGUC	836	1557	UUGUCUGUC GCUGGCGG	837
1318	AGGGACGUC CAACUGUG	838	1571	CGGUGUGUU UCGGUAUG	839
1331	UGUGAGAUC GGAAACCU	840	1572	GGUGUGUUU CGGUAUGU	841
1348	GCUGCGGUC UGCUUAGA	842	1573	GUGUGUUUC GGUAUGUU	843
1353	GGUCUGCUU AGACAAGA	844	1577	GUUUCGGUA UGUUAUUU	845
1354	GUCUGCUUA GACAAGAC	846	1581	CGGUAUGUU AUUUGAGU	847
1372	UGCUGUGUC UGCGUUAC	848	1582	GGUAUGUUA UUUGAGUU	849
1378	GUCUGCGUU ACAUAGGU	850	1584	UAUGUUAUU UGAGUUGC	851
1379	UCUGCGUUA CAUAGGUC	852	1585	AUGUUAUUU GAGUUGCU	853
1383	CGUUACAUA GGUCUCCA	854	1590	AUUUGAGUU GCUCAGAU	855
1387	ACAUAGGUC UCCAGGUU	856	1594	GAGUUGCUC AGAUCUGU	857
1389	AUAGGUCUC CAGGUUUU	858	1599	GCUCAGAUC UGUUAAAA	859
1395	CUCCAGGUU UUGAUCAA	860	1603	AGAUCUGUU AAAAAAA	861
1396	UCCAGGUUU UGAUCAAA	862	1604	GAUCUGUUA AAAAAAA	863

Table VI

nt. Position	Substrate	Seq. ID No.
1397	CCAGGUUUU GAUCAAAU	864
1401	GUUUUGAUC AAAUGGUC	865
1409	CAAAUGGUC CCGUGUCG	866
1416	UCCCGUGUC GUCUUAUA	867
1419	CGUGUCGUC UUAUAGAG	868
1421	UGUCGUCUU AUAGAGCG	869
1422	GUCGUCUUA UAGAGCGA	870
1424	CGUCUUAUA GAGCGAUA	871
1432	AGAGCGAUA GGAGAACG	872
1444	GAACGUGUU GGUCUGUG	873
1448	GUGUUGGUC UGUGGUGU	874
1457	UGUGGUGUA GCUUUGUU	875
1461	GUGUAGCUU UGUUUUUA	876
1462	UGUAGCUUU GUUUUUAU	877
1465	AGCUUUGUU UUUAUUUU	878
1466	GCUUUGUUU UUAUUUUG	879
1467	CUUUGUUUU UAUUUUGU	880
1468	UUUGUUUUU AUUUUGUA	881
1469	UUGUUUUUA UUUUGUAU	882
1471	GUUUUUAUU UUGUAUUU	883
1472	UUUUUAUUU UGUAUUUU	884
1473	UUUUAUUUU GUAUUUUU	885
1476	UAUUUUGUA UUUUUCUG	886
1478	UUUUGUAUU UUUCUGCU	887
1479	UUUGUAUUU UUCUGCUU	888
1480	UUGUAUUUU UCUGCUUU	889
1481	UGUAUUUUU CUGCUUUG	890
1482	GUAUUUUUC UGCUUUGA	891
1487	UUUCUGCUU UGAUGUAC	892
1488	UUCUGCUUU GAUGUACA	893

Table VII: Delta-9 Desaturase HH Ribozyme Sequences

nt. Position	Ribozyme sequence	Seq. ID No.
13	AAGCGGCA CUGAUGA X GAA AGGGCGCG	894
21	GAACGAAC CUGAUGA X GAA AGCGGCAG	895
24	GAGGAACG CUGAUGA X GAA ACAAGCGG	896
25	CGAGGAAC CUGAUGA X GAA AACAAGCG	897
28	GCGCGAGG CUGAUGA X GAA ACGAACAA	898
29	AGCGCGAG CUGAUGA X GAA AACGAACA	899
32	GCGAGCGC CUGAUGA X GAA AGGAACGA	900
38	CUGGUGGC CUGAUGA X GAA AGCGCGAG	901
63	GAGAUUGG CUGAUGA X GAA AUGUGUGU	902
69	CCUCGCGA CUGAUGA X GAA AUUGGGAU	903
71	GCCCUCGC CUGAUGA X GAA AGAUUGGG	904
92	CCGCCGCA CUGAUGA X GAA ACCCUGCU	905
117	GGAGCCGG CUGAUGA X GAA AGCGCGGC	906
118	GGGAGCCG CUGAUGA X GAA AAGCGCGG	907
124	GGGAAGGG CUGAUGA X GAA AGCCGGAA	908
129	CCAAUGGG CUGAUGA X GAA AGGGGAGC	909
130	GCCAAUGG CUGAUGA X GAA AAGGGGAG	910
135	UGGAGGCC CUGAUGA X GAA AUGGGAAG	911
141	CCAUCGUG CUGAUGA X GAA AGGCCAAU	912
154	UUGAGGCG CUGAUGA X GAA AGCGCCAU	913
160	ACGUCGUU CUGAUGA X GAA AGGCGGAG	914
169	CAGAGCGC CUGAUGA X GAA ACGUCGUU	915
175	GAGAGGCA CUGAUGA X GAA AGCGCGAC	916
181	GGCGGGGA CUGAUGA X GAA AGGCAGAG	917
183	GCGGCGGG CUGAUGA X GAA AGAGGCAG	918
193	CGGGCGGC CUGAUGA X GAA AGCGGCGG	919
228	CGGCGACG CUGAUGA X GAA ACCUGCCG	920
229	ACGGCGAC CUGAUGA X GAA AACCUGCC	921
232	GCGACGGC CUGAUGA X GAA ACGAACCU	922
238	AUGGAGGC CUGAUGA X GAA ACGGCGAC	923
243	ACGUCAUG CUGAUGA X GAA AGGCGACG	924
252	AGACGGCG CUGAUGA X GAA ACGUCAUG	925
259	UUGGUGGA CUGAUGA X GAA ACGGCGGA	926
261	CCUUGGUG CUGAUGA X GAA AGACGGCG	927
271	UUAUUCUC CUGAUGA X GAA ACCUUGGU	928
278	UGGCUUCU CUGAUGA X GAA AUUCUCGA	929
288	GAGGAGCA CUGAUGA X GAA AUGGCUUC	930
289	GGAGGAGC CUGAUGA X GAA AAUGGCUU	931
293	CCUUGGAG CUGAUGA X GAA AGCAAAUG	932
296	CUCCCUUG CUGAUGA X GAA AGGAGCAA	933
307	UGGACAUG CUGAUGA X GAA ACCUCCCU	934
313	GUAACCUG CUGAUGA X GAA ACAUGUAC	935
319	GAAUGUGU CUGAUGA X GAA ACCUGGAC	936
320	UGAAUGUG CUGAUGA X GAA AACCUGGA	937
326	UGGCAUUG CUGAUGA X GAA AUGUGUAA	938
327	GUGGCAUU CUGAUGA X GAA AAUGUGUA	939
338	AAUCUUGU CUGAUGA X GAA AGGUGGCA	940
346	AAAAUUUC CUGAUGA X GAA AUCUUGUG	941
352	GACUUGAA CUGAUGA X GAA AUUUCAAU	942
353	CGACUUGA CUGAUGA X GAA AAUUUCAA	943
354	GCGACUUG CUGAUGA X GAA AAAUUUCA	944
355	AGCGACUU CUGAUGA X GAA AAAAUUUC	945
360	CAUCAAGC CUGAUGA X GAA ACUUGAAA	946
364	CAAUCAUC CUGAUGA X GAA AGCGACUU	947

Table VII

nt. Position	Ribozyme sequence	Seq. ID No.
371	UCUAGCCC CUGAUGA X GAA AUCAUCAA	948
377	AUUAUCUC CUGAUGA X GAA AGCCCAAU	949
383	CAAGAUAU CUGAUGA X GAA AUCUCUAG	950
386	CGUCAAGA CUGAUGA X GAA AUUAUCUC	951
388	UGCGUCAA CUGAUGA X GAA AUAUUAUC	952
390	GAUGCGUC CUGAUGA X GAA AGAUAUUA	953
398	UGGCUUGA CUGAUGA X GAA AUGCGUCA	954
400	ACUGGCUU CUGAUGA X GAA AGAUGCGU	955
409	CACUUCUC CUGAUGA X GAA ACUGGCUU	956
419	UGGCUGCC CUGAUGA X GAA ACACUUCU	957
434	CGGGAGGA CUGAUGA X GAA AUCCUGUG	958
435	CCGGGAGG CUGAUGA X GAA AAUCCUGU	959
436	UCCGGGAG CUGAUGA X GAA AAAUCCUG	960
439	GGGUCCGG CUGAUGA X GAA AGGAAAUC	961
453	AUCCUUCA CUGAUGA X GAA AUGCUGGG	962
462	CAUCAUGA CUGAUGA X GAA AUCCUUCA	963
463	UCAUCAUG CUGAUGA X GAA AAUCCUUC	964
464	UUCAUCAU CUGAUGA X GAA AAAUCCUU	965
475	AGCUCCUU CUGAUGA X GAA ACUUCAUC	966
476	GAGCUCCU CUGAUGA X GAA AACUUCAU	967
484	CGUUCUCU CUGAUGA X GAA AGCUCCUU	968
505	UCAUCAGG CUGAUGA X GAA AUUUCCUU	969
515	AACAAAAU CUGAUGA X GAA AUCAUCAG	970
516	AAACAAAA CUGAUGA X GAA AAUCAUCA	971
518	ACAAACAA CUGAUGA X GAA AUAAUCAU	972
519	AACAAACA CUGAUGA X GAA AAUAAUCA	973
520	AAACAAAC CUGAUGA X GAA AAAUAAUC	974
523	ACCAAACA CUGAUGA X GAA ACAAAAUA	975
524	CACCAAAC CUGAUGA X GAA AACAAAAU	976
527	UCCCACCA CUGAUGA X GAA ACAAACAA	977
528	CUCCCACC CUGAUGA X GAA AACAAACA	978
544	UCCUCGGU CUGAUGA X GAA AUCAUGUC	979
545	UUCCUCGG CUGAUGA X GAA AAUCAUGU	980
557	UGUUGGUA CUGAUGA X GAA AGCUUCCU	981
559	UAUGUUGG CUGAUGA X GAA AGAGCUUC	982
567	UAGUCUGG CUGAUGA X GAA AUGUUGGU	983
575	GUUAAGCA CUGAUGA X GAA AGUCUGGU	984
580	AGGGUGUU CUGAUGA X GAA AGCAUAGU	985
581	GAGGGUGU CUGAUGA X GAA AAGCAUAG	986
589	ACACCGUC CUGAUGA X GAA AGGGUGUU	987
598	UCAUCUCU CUGAUGA X GAA ACACCGUC	988
637	CUCGUCCA CUGAUGA X GAA ACAGCCCA	989
638	CCUCGUCC CUGAUGA X GAA AACAGCCC	990
680	GUUGAGCA CUGAUGA X GAA AUCACCAU	991
685	UACUUGUU CUGAUGA X GAA AGCAGAUC	992
693	GGUACAUA CUGAUGA X GAA ACUUGUUG	993
695	GAGGUACA CUGAUGA X GAA AUACUUGU	994
699	CAGUGAGG CUGAUGA X GAA ACAUAUAC	995
703	CUCCCAGU CUGAUGA X GAA AGGUACAU	996
719	CUGCCUCA CUGAUGA X GAA AUCCACCC	997
730	GUCUUCUC CUGAUGA X GAA AUCUGCCU	998
742	AGAUACUG CUGAUGA X GAA AUUGUCUU	999
743	AAGAUACU CUGAUGA X GAA AAUUGUCU	1000
747	CAAUAAGA CUGAUGA X GAA ACUGAAUU	1001
749	GCCAAUAA CUGAUGA X GAA AUACUGAA	1002
751	GAGCCAAU CUGAUGA X GAA AGAUACUG	1003
752	AGAGCCAA CUGAUGA X GAA AAGAUACU	1004

Table VII

nt. Positi n	Ribozyme sequence	Seq. ID N .
754	CCAGAGCC CUGAUGA X GAA AUAAGAUA	1005
759	CCAUUCCA CUGAUGA X GAA AGCCAAUA	1006
770	AGUCCUAG CUGAUGA X GAA AUCCAUUC	1007
773	CUCAGUCC CUGAUGA X GAA AGGAUCCA	1008
785	AUAAGGAU CUGAUGA X GAA AUUCUCAG	1009
788	AAGAUAAG CUGAUGA X GAA AUUAUUCU	1010
791	ACCAAGAU CUGAUGA X GAA AGGAUUAU	1011
792	AACCAAGA CUGAUGA X GAA AAGGAUUA	1012
794	GAAACCAA CUGAUGA X GAA AUAAGGAU	1013
796	AUGAAACC CUGAUGA X GAA AGAUAAGG	1014
800	GUAGAUGA CUGAUGA X GAA ACCAAGAU	1015
801	UGUAGAUG CUGAUGA X GAA AACCAAGA	1016
802	GUGUAGAU CUGAUGA X GAA AAACCAAG	1017
805	GAGGUGUA CUGAUGA X GAA AUGAAACC	1018
807	AGGAGGUG CUGAUGA X GAA AGAUGAAA	1019
813	CUUGGAAG CUGAUGA X GAA AGGUGUAG	1020
816	GCUCUUGG CUGAUGA X GAA AGGAGGUG	1021
817	CGCUCUUG CUGAUGA X GAA AAGGAGGU	1022
834	GUGAGAUG CUGAUGA X GAA AGGUCGCC	1023
835	UGUGAGAU CUGAUGA X GAA AAGGUCGC	1024
838	CCGUGUGA CUGAUGA X GAA AUGAAGGU	1025
840	UCCCGUGU CUGAUGA X GAA AGAUGAAG	1026
857	GGCGUGAC CUGAUGA X GAA AGCAGUGU	1027
860	CUUGGCGU CUGAUGA X GAA ACGAGCAG	1028
873	AGUCGCCA CUGAUGA X GAA AGUCCUUG	1029
874	AAGUCGCC CUGAUGA X GAA AAGUCCUU	1030
882	CAAGCUUU CUGAUGA X GAA AGUCGCCA	1031
883	GCAAGCUU CUGAUGA X GAA AAGUCGCC	1032
889	AUUUGUGC CUGAUGA X GAA AGCUUUAA	1033
898	AUGCCGCA CUGAUGA X GAA AUUUGUGC	1034
907	GAGGCGAU CUGAUGA X GAA AUGCCGCA	1035
910	UCUGAGGC CUGAUGA X GAA AUGAUGCC	1036
915	UCUCAUCU CUGAUGA X GAA AGGCGAUG	1037
942	UCUUGGUG CUGAUGA X GAA ACGCAGUU	1038
952	UUCUCCAC CUGAUGA X GAA AUCUUGGU	1039
966	CGAUCUCA CUGAUGA X GAA ACAGCUUC	1040
967	UCGAUCUC CUGAUGA X GAA AACAGCUU	1041
973	UCAGGGUC CUGAUGA X GAA AUCUCAAA	1042
986	GACCACGG CUGAUGA X GAA ACCAUCAG	1043
994	GCCAGAGC CUGAUGA X GAA ACCACGGU	1044
998	GUCAGCCA CUGAUGA X GAA AGCGACCA	1045
1024	GGCAUUGA CUGAUGA X GAA AUCUUCUU	1046
1026	CAGGCAUU CUGAUGA X GAA AGAUCUUC	1047
1047	GCCCGUCA CUGAUGA X GAA ACAUCAGG	1048
1048	UGCCCGUC CUGAUGA X GAA AACAUCAG	1049
1071	AGUGCUCG CUGAUGA X GAA ACAGCUUG	1050
1072	AAGUGCUC CUGAUGA X GAA AACAGCUU	1051
1080	CCAUGGAG CUGAUGA X GAA AGUGCUCG	1052
1081	ACCAUGGA CUGAUGA X GAA AAGUGCUC	1053
1083	CGACCAUG CUGAUGA X GAA AGAAGUGC	1054
1090	CUCUGCGC CUGAUGA X GAA ACCAUGGA	1055
1102	UAAACGCC CUGAUGA X GAA AGCCUCUG	1056
1108	GCGGUGUA CUGAUGA X GAA ACGCCAAG	1057
1109	GGCGGUGU CUGAUGA X GAA AACGCCAA	1058
1110	UGGCGGUG CUGAUGA X GAA AAACGCCA	1059
1125	UGUCGGCG CUGAUGA X GAA AGUCCCUG	1060
1135	AACUCGAG CUGAUGA X GAA AUGUCGGC	1061

Table VII

nt. Position	Ribozyme sequence	Seq. ID No.
1138	AGGAACUC CUGAUGA X GAA AGGAUGUC	1062
1143	CGACGAGG CUGAUGA X GAA ACUCGAGG	1063
1144	UCGACGAG CUGAUGA X GAA AACUCGAG	1064
1147	CUGUCGAC CUGAUGA X GAA AGGAACUC	1065
1150	CACCUGUC CUGAUGA X GAA ACGAGGAA	1066
1181	ACCCGACA CUGAUGA X GAA ACCAGUCA	1067
1185	CUUCACCC CUGAUGA X GAA ACAGACCA	1068
1212	UGCAAAGG CUGAUGA X GAA AGUCCUGC	1069
1216	AGGGUGCA CUGAUGA X GAA AGGUAGUC	1070
1217	AAGGGUGC CUGAUGA X GAA AAGGUAGU	1071
1225	CUUGAAGC CUGAUGA X GAA AGGGUGCA	1072
1229	GAUUCUUG CUGAUGA X GAA AGCAAGGG	1073
1230	UGAUUCUU CUGAUGA X GAA AAGCAAGG	1074
1237	AGCCUCCU CUGAUGA X GAA AUUCUUGA	1075
1292	CCAGCUGA CUGAUGA X GAA AGGCAGCG	1076
1293	CCCAGCUG CUGAUGA X GAA AAGGCAGC	1077
1294	ACCCAGCU CUGAUGA X GAA AAAGGCAG	1078
1303	CUACCGUA CUGAUGA X GAA ACCCAGCU	1079
1305	CCCUACCG CUGAUGA X GAA AUACCCAG	1080
1310	GACGUCCC CUGAUGA X GAA ACCGUAUA	1081
1318	CACAGUUG CUGAUGA X GAA ACGUCCCU	1082
1331	AGGUUUCC CUGAUGA X GAA AUCUCACA	1083
1348	UCUAAGCA CUGAUGA X GAA ACCGCAGC	1084
1353	UCUUGUCU CUGAUGA X GAA AGCAGACC	1085
1354	GUCUUGUC CUGAUGA X GAA AAGCAGAC	1086
1372	GUAACGCA CUGAUGA X GAA ACACAGCA	1087
1378	ACCUAUGU CUGAUGA X GAA ACGCAGAC	1088
1379	GACCUAUG CUGAUGA X GAA AACGCAGA	1089
1383	UGGAGACC CUGAUGA X GAA AUGUAACG	1090
1387	AACCUGGA CUGAUGA X GAA ACCUAUGU	1091
1389	AAAACCUG CUGAUGA X GAA AGACCUAU	1092
1395	UUGAUCAA CUGAUGA X GAA ACCUGGAG	1093
1396	UUUGAUCA CUGAUGA X GAA AACCUGGA	1094
1397	AUUUGAUC CUGAUGA X GAA AAACCUGG	1095
1401	GACCAUUU CUGAUGA X GAA AUCAAAAC	1096
1409	CGACACGG CUGAUGA X GAA ACCAUUUG	1097
1416	UAUAAGAC CUGAUGA X GAA ACACGGGA	1098
1419	CUCUAUAA CUGAUGA X GAA ACGACACG	1099
1421	CGCUCUAU CUGAUGA X GAA AGACGACA	1100
1422	UCGCUCUA CUGAUGA X GAA AAGACGAC	1101
1424	UAUCGCUC CUGAUGA X GAA AUAAGACG	1102
1432	CGUUCUCC CUGAUGA X GAA AUCGCUCU	1103
1444	CACAGACC CUGAUGA X GAA ACACGUUC	1104
1448	ACACCACA CUGAUGA X GAA ACCAACAC	1105
1457	AACAAAGC CUGAUGA X GAA ACACCACA	1106
1461	UAAAAACA CUGAUGA X GAA AGCUACAC	1107
1462	AUAAAAAC CUGAUGA X GAA AAGCUACA	1108
1465	AAAAUAAA CUGAUGA X GAA ACAAAGCU	1109
1466	CAAAAUAA CUGAUGA X GAA AACAAAGC	1110
1467	ACAAAAUA CUGAUGA X GAA AAACAAAG	1111
1468	UACAAAAU CUGAUGA X GAA AAAACAAA	1112
1469	AUACAAAA CUGAUGA X GAA AAAAACAA	1113
1471	AAAUACAA CUGAUGA X GAA AUAAAAAC	1114
1472	AAAAUACA CUGAUGA X GAA AAUAAAAA	1115
1473	AAAAAUAC CUGAUGA X GAA AAAUAAAA	1116
1476	CAGAAAAA CUGAUGA X GAA ACAAAAUA	1117
1478	AGCAGAAA CUGAUGA X GAA AUACAAAA	1118

Table VH

nt. Positi n	Ribozyme sequence	Seq. ID No.
1479	AAGCAGAA CUGAUGA X GAA AAUACAAA	1119
1480	AAAGCAGA CUGAUGA X GAA AAAUACAA	1120
1481	CAAAGCAG CUGAUGA X GAA AAAAUACA	1121
1482	UCAAAGCA CUGAUGA X GAA AAAAAUAC	1122
1487	GUACAUCA CUGAUGA X GAA AGCAGAAA	1123
1488	UGUACAUC CUGAUGA X GAA AAGCAGAA	1124
1494	ACAGGUUG CUGAUGA X GAA ACAUCAAA	1125
1546	AGACAAAG CUGAUGA X GAA ACGGCAUG	1126
1549	GACAGACA CUGAUGA X GAA AGUACGGC	1127
1550	CGACAGAC CUGAUGA X GAA AAGUACGG	1128
1553	CAGCGACA CUGAUGA X GAA ACAAAGUA	1129
1557	CCGCCAGC CUGAUGA X GAA ACAGACAA	1130
1571	CAUACCGA CUGAUGA X GAA ACACACCG	1131
1572	ACAUACCG CUGAUGA X GAA AACACACC	1132
1573	AACAUACC CUGAUGA X GAA AAACACAC	1133
1577	AAAUAACA CUGAUGA X GAA ACCGAAAC	1134
1581	ACUCAAAU CUGAUGA X GAA ACAUACCG	1135
1582	AACUCAAA CUGAUGA X GAA AACAUACC	1136
1584	GCAACUCA CUGAUGA X GAA AUAACAUA	1137
1585	AGCAACUC CUGAUGA X GAA AAUAACAU	1138
1590	AUCUGAGC CUGAUGA X GAA ACUCAAAU	1139
1594	ACAGAUCU CUGAUGA X GAA AGCAACUC	1140
1599	UUUUAACA CUGAUGA X GAA AUCUGAGC	1141
1603	UUUUUUUU CUGAUGA X GAA ACAGAUCU	1142
1604	UUUUUUUU CUGAUGA X GAA AACAGAUC	1143

Where "X" represents stem II region of a HH ribozyme (Hertel et al., 1992 *Nucleic Acids Res.* 20 3252). The length of stem II may be ≥ 2 base-pairs.

Table VIII

Table VIII: Delta-9 Desaturase Hairpin Ribozyme and Substrate Sequences

nt. Position	Ribozyme	Seq. ID No.	Substrate	Seq. ID No.
14	GAACAAGC AGAA GAGGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1144	GCCCUCU GCC GCUUGUUC	1145
17	AACGAACA AGAA GCAGAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1146	CUCUGCC GCU UGUUCGUU	1147
108	GGAAGCGC AGAA GCCGCC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1148	GGCGGCG GCC GCGCUUCC	1149
120	GGAAGGGG AGAA GGAAGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1150	GCUUCCG GCU CCCCUUCC	1151
155	GUCGUUGA AGAA GAGCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1152	GCGCUCC GCC UCAACGAC	1153
176	CGGGGAGA AGAA GAGCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1154	GCGCUCU GCC UCUCCCCG	1155
186	CGGCGAGC AGAA GGGAGA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1156	UCUCCCC GCC GCUCGCCG	1157
189	GGGCGGCG AGAA GCGGGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1158	CCCCGCC GCU CGCCGCCC	1159
196	CGGCGGCG AGAA GCGAGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1160	GCUCGCC GCC CGCCGCCG	1161
200	GCGGCGGC AGAA GGCGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1162	GCCGCCC GCC GCCGCCGC	1163
203	GCGGCGGC AGAA GCGGGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1164	GCCCGCC GCC GCCGCCGC	1165
206	GCUGCGGC AGAA GCGGCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1166	CGCCGCC GCC GCCGCAGC	1167
209	GCUGCUGC AGAA GCGGCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1168	CGCCGCC GCC GCAGCAGC	1169
235	AUGGAGGC AGAA GCGACG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1170	CGUCGCC GUC GCCUCCAU	1171
253	GUGGAGAC AGAA GACGUC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1172	GACGUCC GCC GUCUCCAC	1173
256	UUGGUGGA AGAA GCGGAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1174	GUCCGCC GUC UCCACCAA	1175
406	CACUUCUC AGAA GGCUUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1176	CAAGCCA GUC GAGAAGUG	1177
442	GAUGCUGG AGAA GGGAGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1178	CCUCCCG GAC CCAGCAUC	1179
508	AAAUAAUC AGAA GGGAUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1180	AAUCCCU GAU GAUUAUUU	1181
570	UAAGCAUA AGAA GGUAUG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1182	CAUACCA GAC UAUGCUUA	1183
625	ACAGCCCA AGAA GUGGGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1184	CCCCACU GCC UGGGCUGU	1185
634	CUCGUCCA AGAA GCCCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1186	CUGGGCU GUU UGGACGAG	1187
655	UUCUCCUC AGAA GUCCAU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1188	AUGGACU GCU GAGGAGAA	1189
681	ACUUGUUG AGAA GAUCAC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1190	GUGAUCU GCU CAACAAGU	1191
726	UCUUCUCA AGAA GCCUCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1192	UGAGGCA GAU UGAGAAGA	1193
853	GCGUGACG AGAA GUGUUC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1194	GAACACU GCU CGUCACGC	1195
916	CGCUUCUC AGAA GAGGCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1196	CGCCUCA GAU GAGAAGCG	1197

Table VIII

nt. Position	Ribozyme	Seq. ID No.	Substrate	Seq. ID No.
963	CGAUCUCA AGAA GCUUCU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1198	AGAAGCU GUU UGAGAUCG	1199
979	ACGGUACC AGAA GGGUCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1200	CGACCCU GAU GGUACCGU	1201
1033	AUCAGGUG AGAA GGCAUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1202	AAUGCCU GCC CACCUGAU	1203
1041	CGUCAAAC AGAA GGUGGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1204	CCCACCU GAU GUUUGACG	1205
1068	AGUGCUCG AGAA GCUUGU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1206	ACAAGCU GUU CGAGCACU	1207
1173	ACAGACCA AGAA GGCUCG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1208	CGAGCCU GAC UGGUCUGU	1209
1182	CUUCACCC AGAA GACCAG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1210	CUGGUCU GUC GGGUGAAG	1211
1287	AGCUGAAA AGAA GCGUGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1212	GCACGCU GCC UUUCAGCU	1213
1295	GUAUACCC AGAA GAAAGG ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1214	CCUUUCA GCU GGGUAUAC	1215
1339	CAGACCGC AGAA GGUUUC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1216	GAAACCU GCU GCGGUCUG	1217
1345	UCUAAGCA AGAA GCAGCA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1218	UGCUGCG GUC UGCUUAGA	1219
1349	CUUGUCUA AGAA GACCGC ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1220	GCGGUCU GCU UAGACAAG	1221
1364	GCAGACAC AGAA GGUCUU ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1222	AAGACCU GCU GUGUCUGC	1223
1483	UACAUCAA AGAA GAAAAA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1224	UUUUUCU GCU UUGAUGUA	1225
1554	CCGCCAGC AGAA GACAAA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1226	UUUGUCU GUC GCUGGCGG	1227
1595	UUUAACAG AGAA GAGCAA ACCAGAGAAACACACGUUGUGGUACAUUACCUGGUA	1228	UUGCUCA GAU CUGUUAAA	1229

Table IX

Table IX: Cleavage of D elta-9 Desaturase RNA by HH Ribozymes

	Percent Cleaved			
	20°C		26°C	
nt. Position	10 min	120 min	10 min	120 min
183	6.3	7.0	10.45	11.8
252	25.2	51.2	33.1	52.9
259	20.3	41.3	24.8	44.0
271	17.2	52.4	21.5	56.3
278	9.9	25.7	13.3	33.6
307	10.3	24.2	9.2	32.4
313	16.9	43.0	23.8	53.4
320	10.6	23.6	15.0	31.3
326	5.7	14.6	8.0	17.1
338	10.0	17.5	10.4	12.9
353	10.2	11.3	10.7	14.7
390	8.6	8.9	7.8	9.8
419	6.3	10.1	5.8	10.9
453	7.3	29.0	8.0	33.8
484	7.8	28.9	6.9	29.2
545	4.8	8.5	3.6	8.9
773	4.5	11.5	4.4	8.9
1024	11.9	17.1	13.3	23.8
1026	11.6	12.6	13.1	17.2
1237	23.1	32.4	13.8	28.6

TABLE X:

Construct Number	*Targets Blasted*	*Isolates Recovered*	*Greenhouse Lines*	*Plants Produced*
RPA85	231	70	13	161
RPA113	292	82	9	116
RPA114	244	35	12	152
RPA115	285	42	11	165
RPA118	268	38	10	125
RPA119	301	67	11	135
Totals	1621	334	66	854

Table XI Stearic acid levels in leaves from plants transformed with active and inactive ribozymes compared to control leaves.

Stearic Acid in Leaves Transformed with Active and Inactive Ribozymes (Percentage of total plants with certain levels of leaf stearic acid)			
Stearic Acid	Ribozyme Actives (428 plants from 35 lines)	Ribozyme Inactives (406 plants from 31 lines)	Controls (122 plants)
> 3%	7%	3%	2%
> 5%	2%	0	0
> 10%	0	0	0

Table XII Inheritance of the high stearic acid trait in leaves from crosses of high stearic acid plants.

Inheritance of high stearate in leaves.			
Cross	R1 Plants with Normal Leaf Stearate	R1 Plants with High Leaf Stearate	% of Plants with High Stearate
RPA85-15.06 x RPA85-15.12	6	3	33%
RPA85-15.07 self	5	5	50%
RPA85-15.10 self	8	2	20%
OQ414 x RPA85-15.06	5	3	38%
OQ414 x RPA85-15.11	6	4	40%

Table XIII Comparis_n _f fatty acid composition of embryogenic callus, somatic embryos and zygotic embryos.

Tissue and/or Media Treatment	Fatty Acid Composition					% Lipid of Fresh Weight
	C16:0	C18:0	C18:1	C18:2	C18:3	
embryogenic callus	19.4 +/- 0.9	1.1 +/- 0.1	6.2 +/- 2.0	55.7 +/- 3.1	8.8 +/- 2.0	0.4 +/- 0.1
somatic embryo grown on MS + 6% sucrose + 10 mM ABA	12.6 +/- 0.7	1.6 +/- 0.8	18.2 +/- 4.9	60.7 +/- 5.1	1.9 +/- 0.3	4.0 +/- 1.1
zygotic embryo 12 days after pollination	14.5 +/- 0.4	1.1 +/- 0.1	18.5 +/- 1.0	60.2 +/- 1.5	1.4 +/- 0.2	3.9 +/- 0.6

Table XIV: GBSS activity, amylose content, and Southern analysis results of selected Ribozyme Lin

Line	GBSS activity (Units/mg starch)	Amylose Content (%)	Southern
RPA63.0283	321.5 ± 31.2	23.3 ± 0.5	-
RPA63.0236	314.6 ± 9.2	27.4 ± 0.3	-
RPA63.0219	299.8 ± 10.4	21.5 ± 0.3	-
RPA63.0314	440.4 ± 17.1	19.1 ± 0.8	-
RPA63.0316	346.5 ± 8.5	17.9 ±0.5	-
RPA63.0311	301.5 ± 17.4	19.5 ± 0.4	-
RPA63.0309	264.7 ± 19	21.7 ± 0.1	+
RPA63.0218	190.8 ± 7.8	21.0 ± 0.3	+
RPA63.0209	203 ± 2.4	22.6 ± 0.6	+
RPA63.0306	368.2 ± 7.5	19.0 ± 0.4	-
RPA63.0210	195.1 ± 7	22.1 ± 0.2	+

Claims

1. An enzymatic nucleic acid molecule with RNA cleaving activity, wherein said nucleic acid molecule modulates the expression of a plant gene.

2. The enzymatic nucleic acid molecule of claim 1, wherein said plant is a monocotyledon.

3. The enzymatic nucleic acid molecule of claim 1, wherein said plant is a dicotyledon.

4. The enzymatic nucleic acid molecule of claim 1, wherein said plant is a gymnosperm.

5. The enzymatic nucleic acid molecule of claim 1, wherein said plant is an angiosperm.

6. The enzymatic nucleic acid molecule of claim 1, wherein said nucleic acid is in a hammerhead configuration.

7. The enzymatic nucleic acid molecule of claim 1, wherein said nucleic acid is in a hairpin configuration.

8. The enzymatic nucleic acid molecule of claim 1, wherein said nucleic acid is in a hepatitis Δ virus, group I intron, group II intron, VS nucleic acid or RNaseP nucleic acid configuration.

9. The enzymatic nucleic acid of any of claims 1-8, wherein said nucleic acid comprises between 12 and 100 bases complementary to RNA of said gene.

10. The enzymatic nucleic acid of any of claims 1-8, wherein said nucleic acid comprises between 14 and 24 bases complementary to RNA of said gene.

11. The enzymatic nucleic acid of claim 6, wherein said hammerhead comprises a stem II region of length greater than on equal to two base-pairs.

12 The enzymatic nucleic acid of claim 7, wherein said hairpin comprises a stem II region of length between three and seven base-pairs.

13. The enzymatic nucleic acid of claim 7, wherein said hairpin comprises a stem IV region of length greater than or equal to two base-pairs.

14. The enzymatic nucleic acid of claim 2, wherein said monocotyledon plant is selected from a group consisting of maize, rice, wheat, and barley.

15. The enzymatic nucleic acid of claim 3, wherein said dicotyledon plant is selected from a group consisting of canola, sunflower, safflower, soybean, cotton, peanut, olive, sesame, cuphea, flax, jojoba, and grape.

16. The enzymatic nucleic acid of claim 1, wherein said gene is involved in fatty acid biosynthesis in said plant.

17. The enzymatic nucleic acid of claim 16, wherein said gene is Δ-9 desaturase.

18. The enzymatic nucleic acid of any of claims 16 or 17, wherein said plant is selected from a group consisting of maize, canola, flax, sunflower, cotton, peanuts, safflower, soybean and rice.

19. The enzymatic nucleic acid of claim 1, wherein said gene is involved in starch biosynthesis in said plant.

20. The enzymatic nucleic acid of claim 19, wherein said gene is granule bound starch synthase.

21. The enzymatic nucleic acid of any of claims 19 or 20, wherein said plant is selected from a group consisting of maize, potato, wheat, and cassava.

22. The enzymatic nucleic acid of claim 1, wherein said gene is involved in caffeine synthesis.

23. The enzymatic nucleic acid of claim 22, wherein said gene is selected from a group consisting of 7-methylguanosine and 3-methyl transferase.

24. The enzymatic nucleic acid of any of claims 22 or 23, wherein said plant is a coffee plant.

25. The enzymatic nucleic acid of claim 1, wherein said gene is involved in nicotine production in said plant.

26. The enzymatic nucleic acid of claim 25, wherein said gene is selected from a group consisting of *N*-methylputrescine oxidase and putrescine *N*-methyl transferase.

27. The enzymatic nucleic acid of any of claims 25 or 26, wherein said plant is a tobacco plant.

28. The enzymatic nucleic acid of claim 1, wherein said gene is involved in fruit ripening process in said plant.

29. The enzymatic nucleic acid of claim 28, wherein said gene is selected from a group consisting of ethylene-forming enzyme, pectin methyltransferase, pectin esterase, polygalacturonase, 1-aminocyclopropane carboxylic acid (ACC) synthase, and ACC oxidase.

30. The enzymatic nucleic acid of any of claims 28 or 29, wherein said plant is selected from a group consisting of apple, tomato, pear, plum and peach.

31. The enzymatic nucleic acid of claim 1, wherein said gene is involved in flower pigmentation in said plant.

32. The enzymatic nucleic acid of claim 31, wherein said gene is selected from a group consisting of chalcone synthase, chalcone flavanone isomerase, phenylalanine ammonia lyase, dehydroflavonol hydroxylases, and dehydroflavonol reductase.

33. The enzymatic nucleic acid of any of claims 31 or 32, wherein said plant is selected from a group consisting of rose, petunia, chrysanthamum, and marigold.

34. The enzymatic nucleic acid of claim 1, wherein said gene is involved in lignin production in said plant.

35. The enzymatic nucleic acid of claim 34, wherein said gene is selected from a group consisting of *O*-methyltransferase, cinnamoyl-CoA:NADPH reductase and cinnamoyl alcohol dehydrogenase.

36. The enzymatic nucleic acid of any of claims 34 or 35, wherein said plant is selected from a group consisting of tobacco, aspen, poplar, and pine.

37. A nucleic acid fragment comprising a cDNA sequence coding for maize Δ-9 desaturase, wherein said sequence is represented by the sequence I.D. No. 1.

38. The enzymatic nucleic acid molecule of claim 17, wherein said nucleic acid specifically cleaves any of sequences defined in Table VI, wherein said nucleic acid is in a hammerhead configuration.

39. The enzymatic nucleic acid molecule of claim 17, wherein said nucleic acid specifically cleaves any of sequences defined in Table VIII, wherein said nucleic acid is in a hairpin configuration.

40. The enzymatic nucleic acid molecule of any of claims 38 or 39, consisting essentially of one or more sequences selected from the group shown in Tables VII and VIII.

41. The enzymatic nucleic acid molecule of claim 20, wherein said nucleic acid specifically cleaves any of sequences defined in Table IIIA, wherein said nucleic acid is in a hammerhead configuration.

42. The enzymatic nucleic acid molecule of claim 20, wherein said nucleic acid specifically cleaves any of sequences defined in Tables VA and VB, wherein said nucleic acid is in a hairpin configuration.

43. The enzymatic nucleic acid molecule of any of claims 41 or 42, consisting essentially of one or more sequences selected from the group shown in Tables IIIB, IV, VA and VB.

44. The enzymatic nucleic acid molecule of claim 41, consisting essentially of sequences defined as any of SEQ. I.D. NOS. 2-24.

45. A plant cell comprising the enzymatic nucleic acid molecule of any of claims 1-8, 11-17, 19-20, 22-23, 25-26, 28-29, 31-32, 34-35, 37-39, 41-42 or 44.

46. A transgenic plant and the progeny thereof, comprising the enzymatic nucleic acid molecule of any of claims 1-8, 11-17, 19-20, 22-23, 25-26, 28-29, 31-32, 34-35, 37-39, 41-42 or 44.

47. An expression vector comprising nucleic acid encoding the enzymatic nucleic acid molecule of any of claims 1-8, 11-17, 19-20, 22-23, 25-26, 28-29, 31-32, 34-35,

37-39, 41-42 or 44, in a manner which allows expression and/or delivery of that enzymatic nucleic acid molecule within a plant cell.

48. An expression vector comprising nucleic acid encoding a plurality of enzymatic nucleic acid molecules of any of claims 1-8, 11-17, 19-20, 22-23, 25-26, 28-29, 31-32, 34-35, 37-39, 41-42 or 44, in a manner which allows expression and/or delivery of said enzymatic nucleic acid molecules within a plant cell.

49. A plant cell comprising the expression vector of claim 47.

50. A plant cell comprising the expression vector of claim 48.

51. A transgenic plant and the progeny thereof, comprising the expression vector of claim 47.

52. A transgenic plant and the progeny thereof, comprising the expression vector of claim 48.

53. A plant cell comprising the enzymatic nucleic acid of any of claims 16 or 17.

54. The plant cell of claim 53, wherein said cell is a maize cell.

55. The plant cell of claim 53, wherein said cell is a canola cell.

56. A transgenic plant and the progeny thereof, comprising the enzymatic nucleic acid of any of claims 16 or 17.

57. The transgenic plant and the progeny thereof of claim 56, wherein said plant is a maize plant.

58. The transgenic plant and the progeny thereof of claim 56, wherein said plant is a canola plant.

59. A plant cell comprising the enzymatic nucleic acid of any of claims 19 or 20.

60. The plant cell of claim 59, wherein said cell is a maize cell.

61. A transgenic plant and the progeny thereof, comprising the enzymatic nucleic acid of any of claims 19 or 20.

62. The transgenic plant and progeny thereof of claim 61, wherein said plant is a maize plant.

63. A method for modulating expression of an gene in a plant by administering to said plant the enzymatic nucleic acid molecule of any of claims 1-8.

64. The method of claim 63, wherein said plant is a monocot plant.

65. The method of claim 63, wherein said plant is a dicot plant.

66. The method of claim 63, wherein said plant is a gymnosperm.

67. The method of claim 63, wherein said plant is an angiosperm.

68. The method of claim 63, wherein said gene is Δ-9 desaturase.

69. The method of claim 68, wherein said plant is a maize plant.

70. The method of claim 68, wherein said plant is a canola plant.

71. The method of claim 63, wherein said gene is granule bound starch synthase.

72. The method of claim 71, wherein said plant is a maize plant.

73. The expression vector of claim 47, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) a gene encoding at least one said enzymatic nucleic acid molecule; and,

wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said enzymatic molecule within said plant cell.

74. The expression vector of claim 47, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an open reading frame;

d) a gene encoding at least one said enzymatic nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and,

wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said enzymatic molecule within said plant cell.

75. The expression vector of claim 47, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) a gene encoding at least one said enzymatic nucleic acid molecule; and,

wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said enzymatic molecule within said plant cell.

76. The expression vector of claim 47, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) an open reading frame;

e) a gene encoding at least one said enzymatic nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and,wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said enzymatic molecule within said plant cell.

77. The enzymatic nucleic acid of Claim 1, wherein said plant is selected from the group consisting of maize, rice, soybeans, canola, alfalfa, cotton, wheat, barley, sunflower, flax and peanuts.

78. A transgenic plant comprising nucleic acids encoding for an enzymatic nucleic acid molecule with RNA cleaving activity, wherein said nucleic acid molecule modulates the expression of a gene in said plant.

79. The transgenic plant of Claim 78, wherein said Plant is selected from the group consisting of maize, rice, soybeans, canola, alfalfa, cotton, wheat, barley, sunflower, flax and peanuts.

80. The transgenic plant of Claim 78, wherein said gene is granule bound starch synthase (GBSS).

81. The transgenic plant of Claim 78, wherein said gene is delta 9 desaturase.

82. The transgenic plant of Claim 78, wherein the plant is transformed with *Agrobacteriurn,* bombarding with DNA coated microprojectiles, whiskers, or electroporation.

83. The transgenic plant of Claim 82, wherein said bombarding with DNA coated microprojectiles is done with the gene gun.

84. The transgenic plant of any of Claims 78 or 82, wherein said plant contains a selectable marker selected from the group consisting of chlorosulfuron, hygromycin, bar gene, bromoxynil, and kanamycin and the like.

85. The transgenic plant of any of Claims 78 or 82, wherein said nucleic acid is operably linked to a promoter selected from the group consisting of octopine synthetase, the nopaline synthase, the manopine synthetase, cauliflower mosaic virus (35S); ribulose-1, 6-biphosphate (RUBP) carboxylase small subunit (ssu), the beta-conglycinin, the phaseolin promoter, napin, gamma zein, globulin, the ADH promoter, heat-shock, actin, and ubiquitin.

86. The transgenic plant of Claim 78, said enzymatic nucleic acid molecule is in a hammerhead, hairpin, hepatitis Δ virus, group I intron, group II intron, VS nucleic acid or RNaseP nucleic acid configuration

87. The transgenic plant of Claim 86, wherein said enzymatic nucleic acid with RNA cleaving activity encoded as a monomer.

88. The transgenic plant of Claim 86, wherein said enzymatic nucleic acid with RNA cleaving activity encoded as a multimer.

89. The transgenic plant of Claim 78, wherein the nucleic acids encoding for said enzymatic nucleic acid molecule with RNA cleaving activity is operably linked to the 3' end of an open reading frame.

90. The transgenic plant of Claim 78, wherein said gene is an endogenous gene.

91. A transgenic maize plant comprising in the 5' to 3' direction of transcription:

a promoter functional in said plant;

a double strand DNA (dsDNA) ~~sequence encoding for a delta-9 gene~~ of SEQ ID. No. 1, wherein transcribed strand of said dsDNA is complementary to RNA endogenous to said plant; and

a termination region functional in said plant.

92. A transgenic maize plant comprising in the 5' to 3' direction of transcription,

a promoter functional in said plant;

a double strand DNA (dsDNA) sequence encoding for a granule bound starch synthase (GBSS) gene of SEQ ID NO. 25, wherein transcribed strand of said dsDNA is complementary to RNA endogenous to said plant; and

a termination region functional in said plant.

93. The enzymatic nucleic acid molecule of claim 1, wherein said gene is an endogenous gene.

94. The method of modulating expression of a gene of claim 63, wherein siad gene is an endogenous gene.

95. The vector of Figure 42, wherein said vector is employed for transformation of a plant cell.

Figure 1. Hammerhead Ribozyme

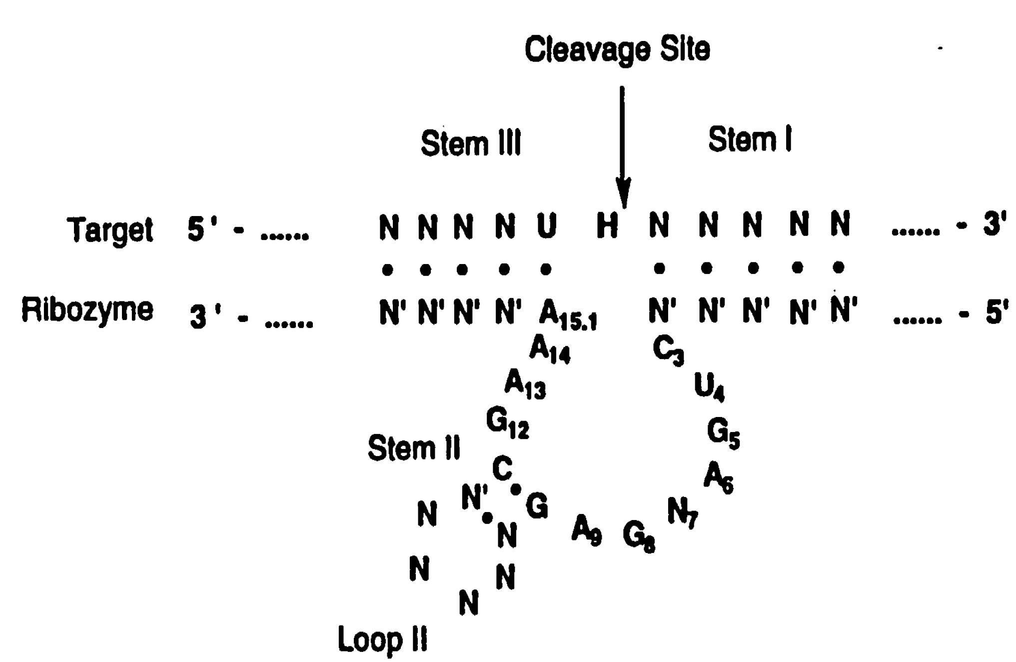


Figure 2. Hammerhead Ribozyme Substrate Motifs

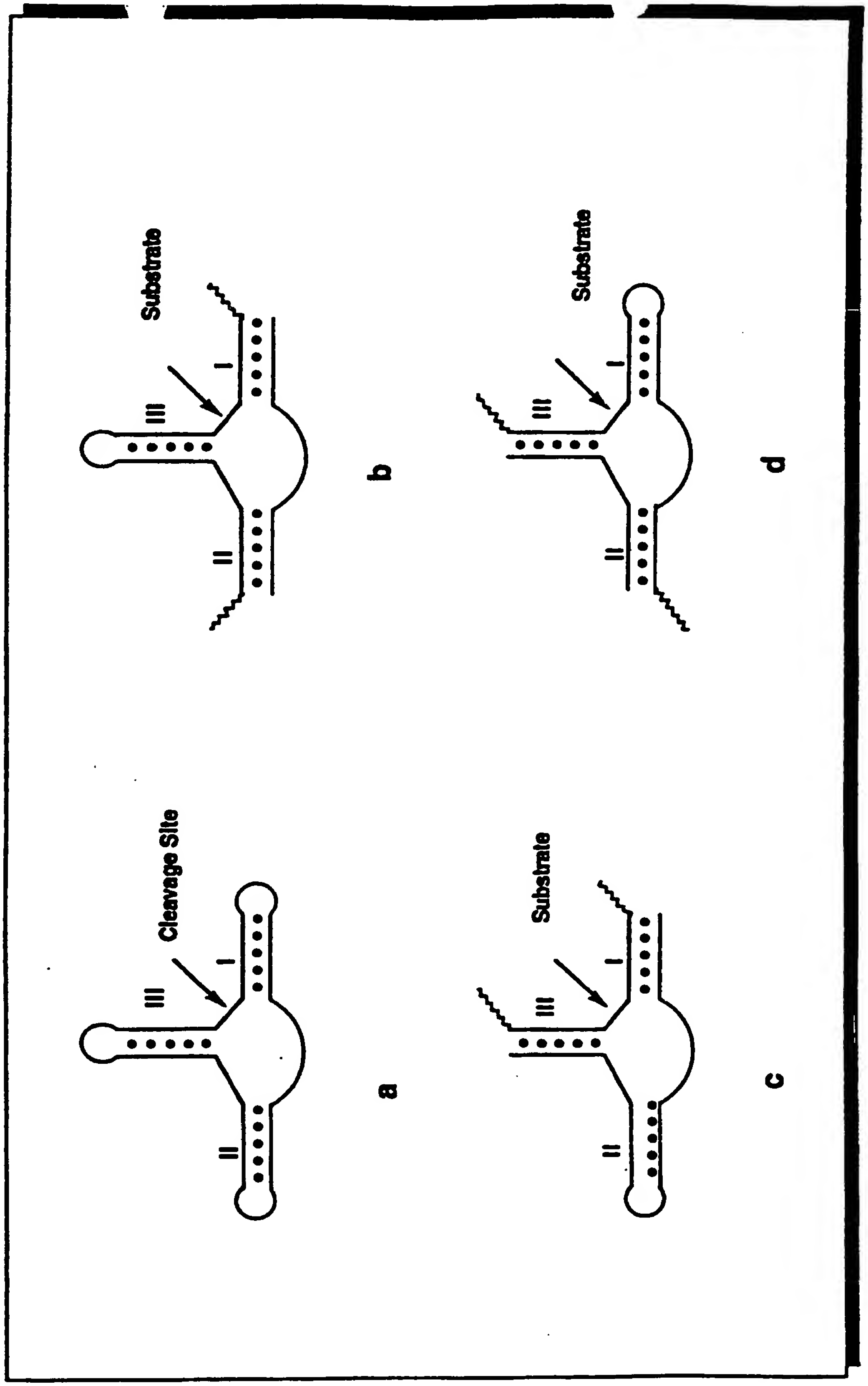
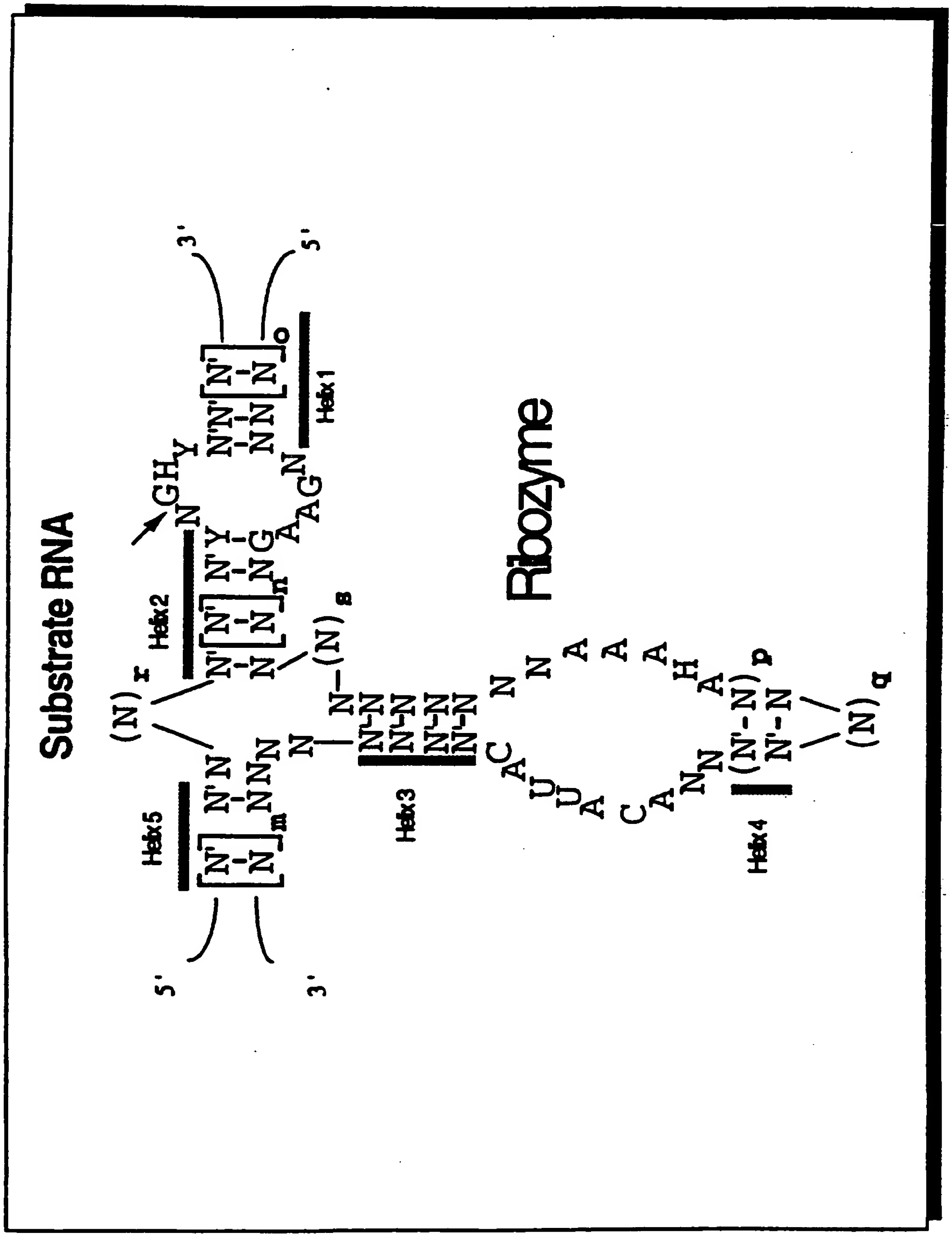


Figure 3. Hairpin Ribozyme



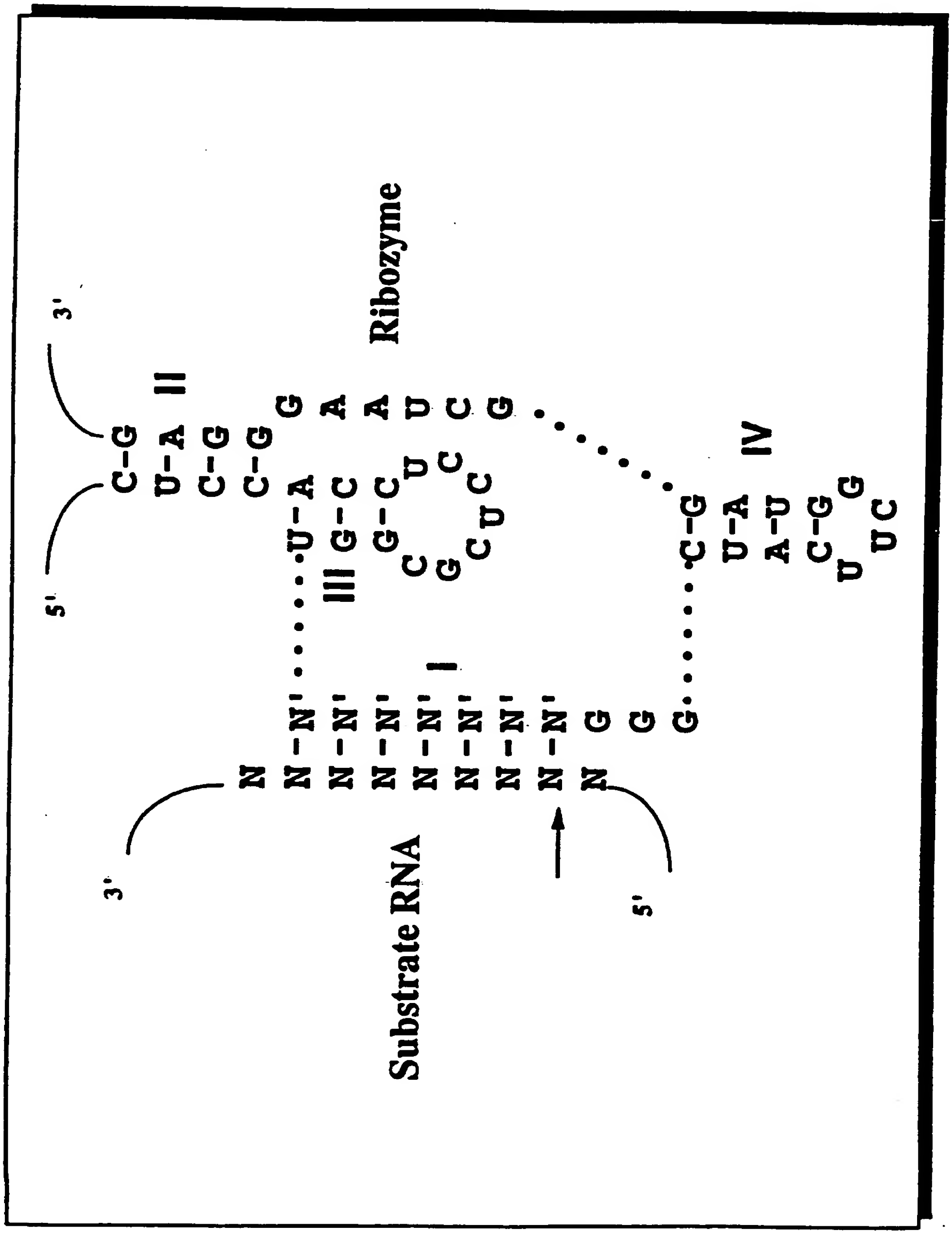


Figure 4. Hepatitis Delta Virus (HDV) Ribozyme

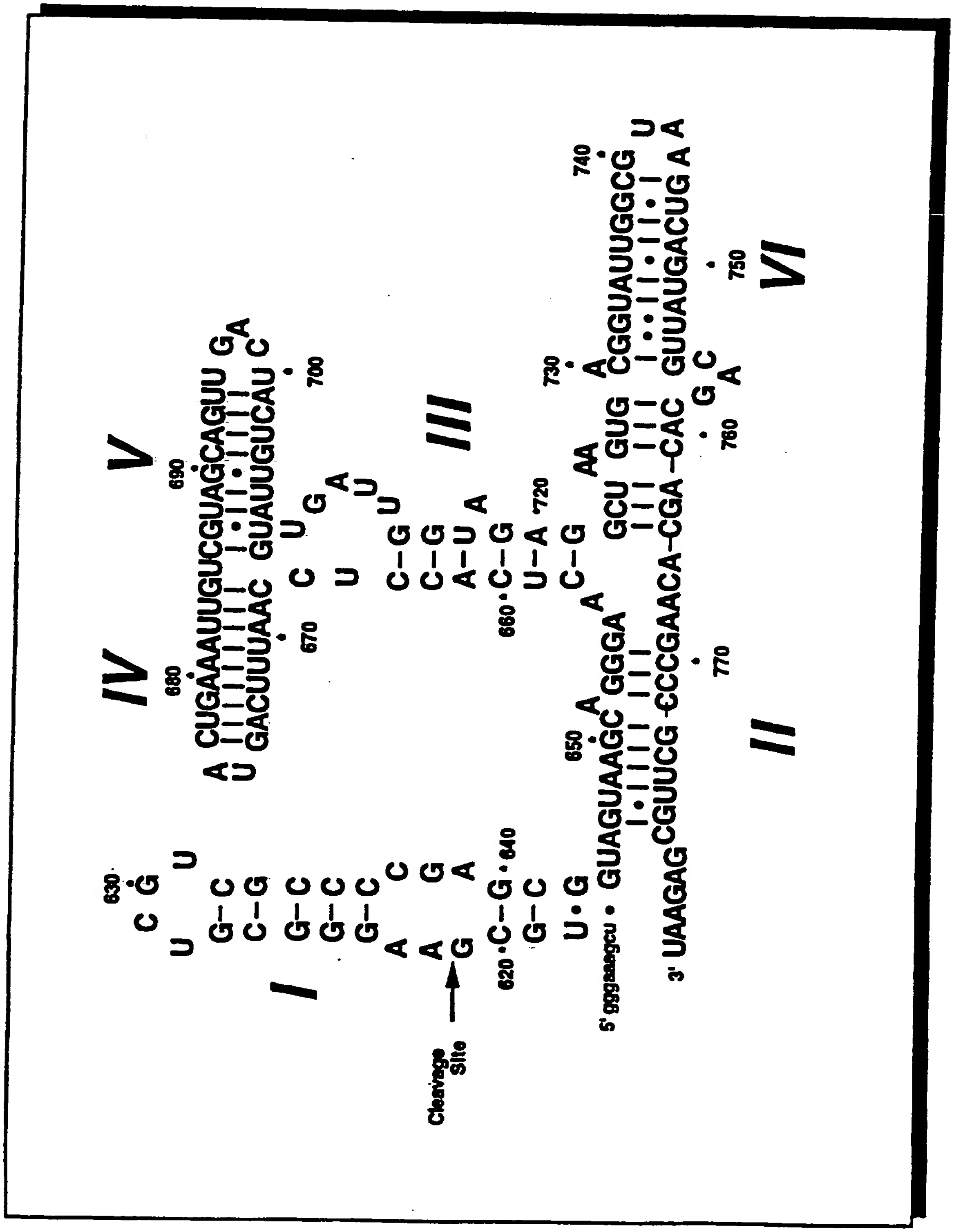
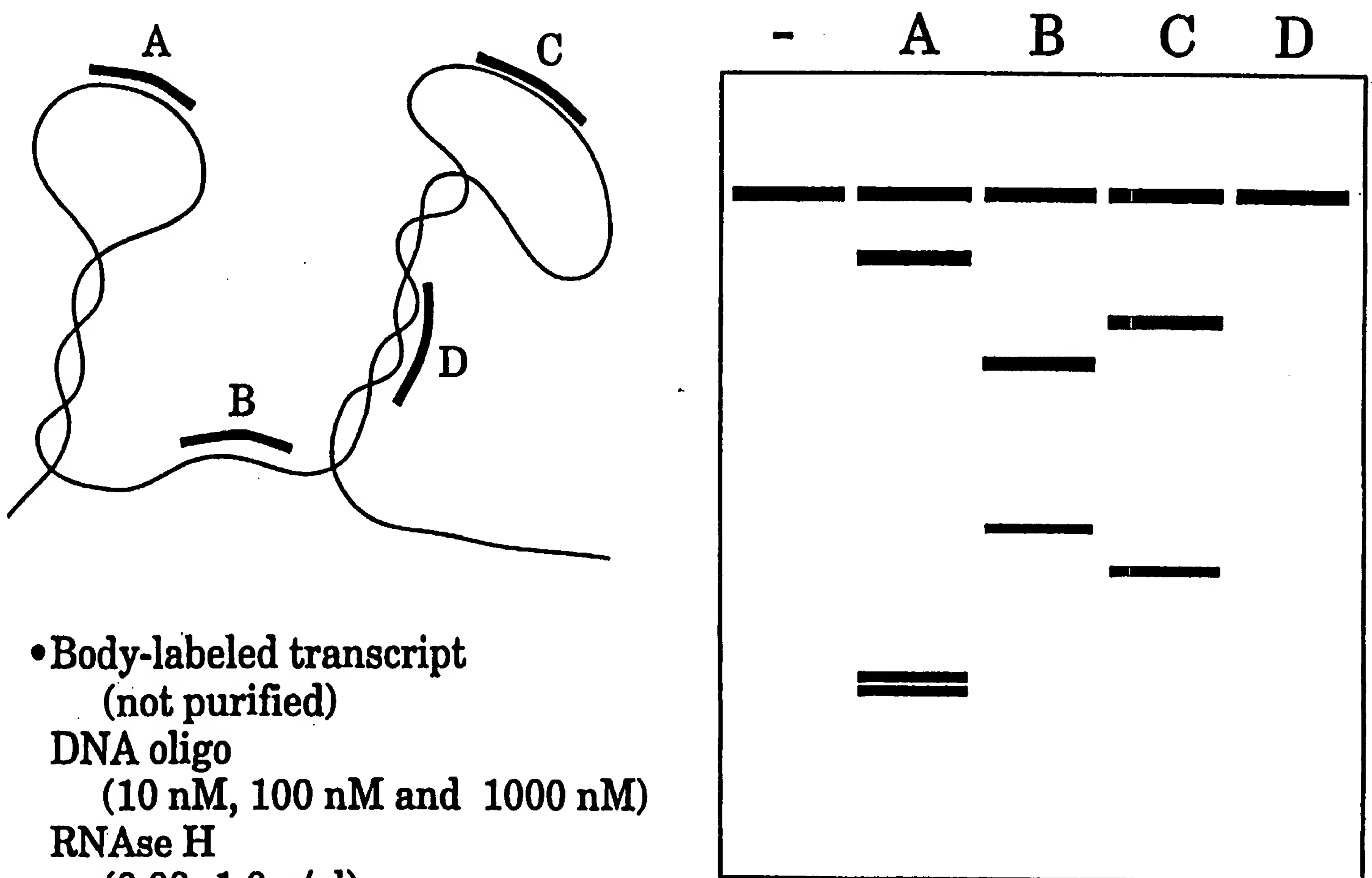


Figure 5. Neurospora VS Ribozyme

Figure 6: RNase H Assay



- Body-labeled transcript
  (not purified)
  DNA oligo
  (10 nM, 100 nM and 1000 nM)
  RNAse H
  (0.08 -1.0 u/μl)
- 37°C, 10 min

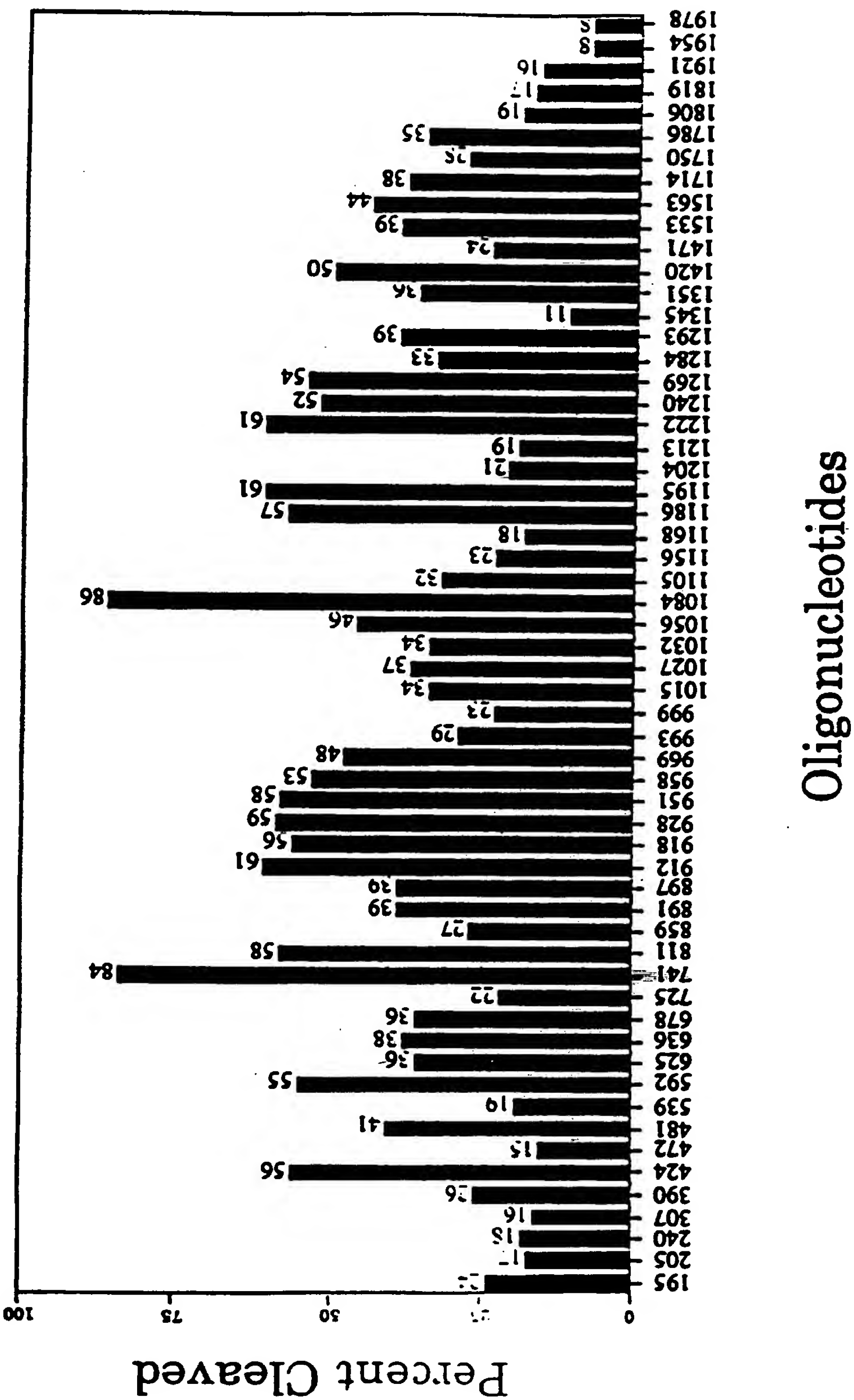


Figure 7: RNase H Accessibility of GBSS mRNA

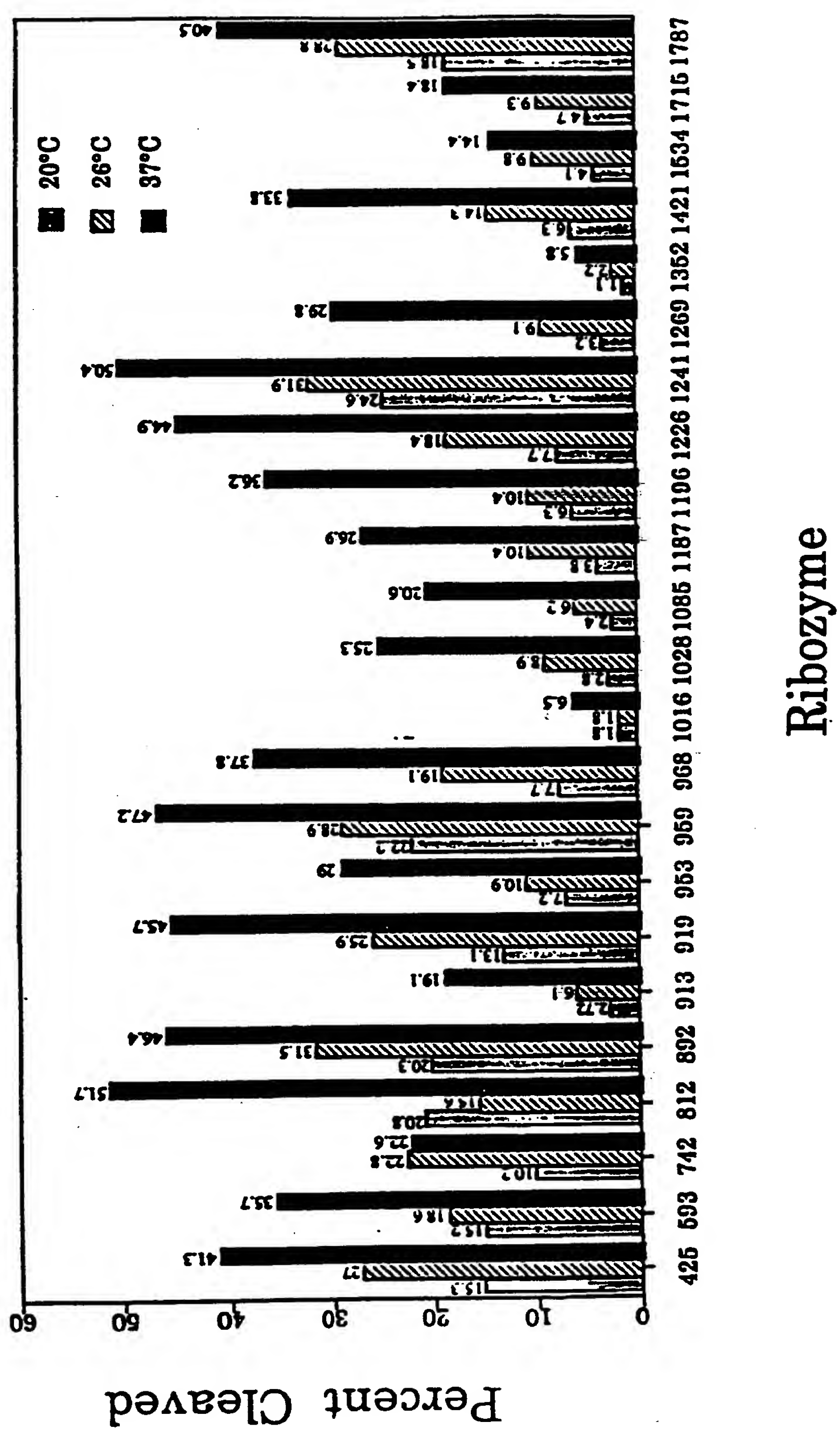


Figure 8: Cleavage of GBSS RNA by HH Ribozymes

Figure 9: Cleavage of GBSS RNA by Multiple HH Ribozymes

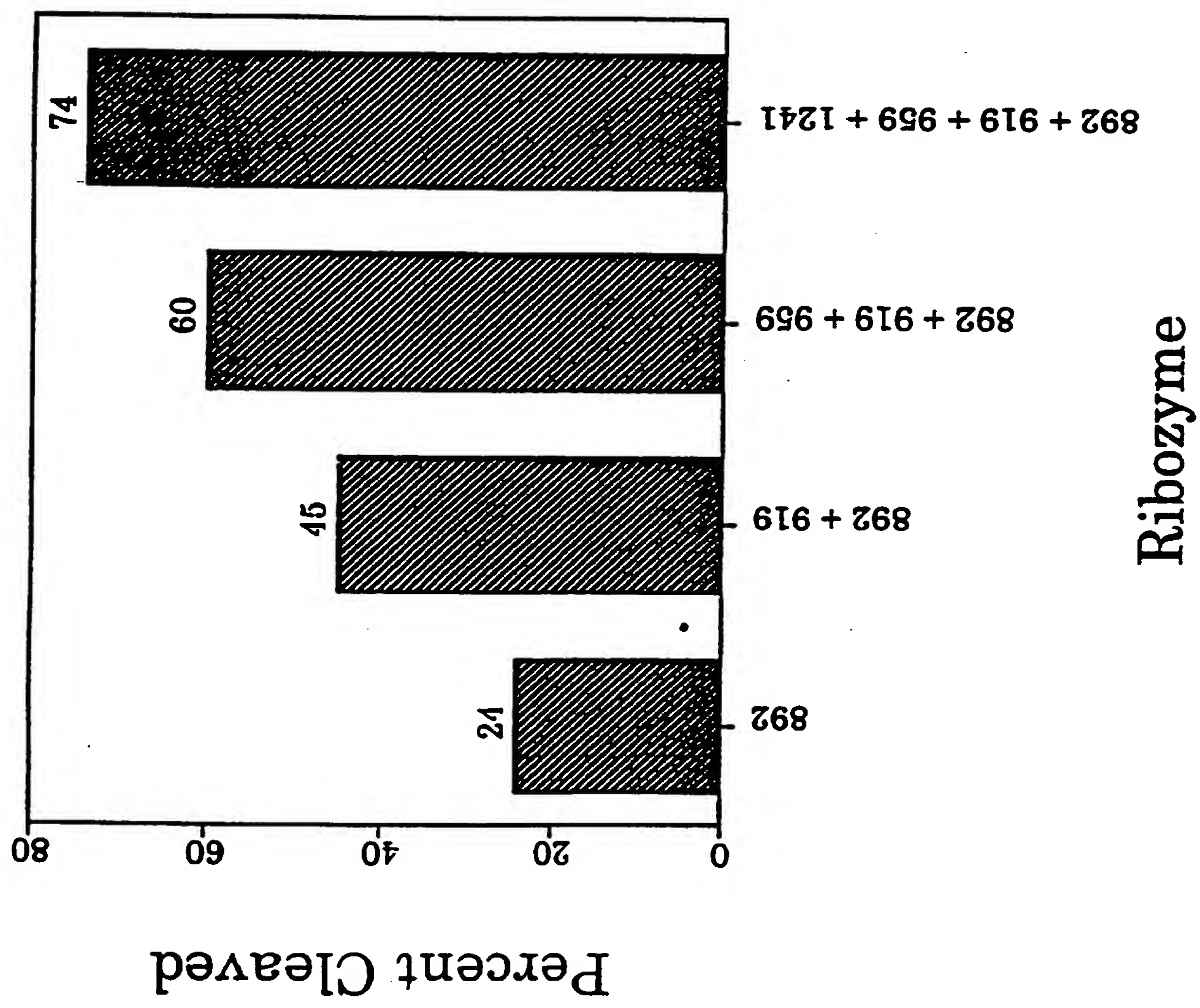


Figure 10

1

Figure 10: Delta-9 Desaturase cDNA Sequence (Seq. I.D. No. 1)

Sequence Range: 1 to 1621

```
         5         10         15         20         25         30         35         40         45         50         55         60
CGCACGCGCC CTCTGCCGCT TGTTCGTTCC TCGCGCTCGC CACCAGGCAC CACCACACAC

        65         70         75         80         85         90         95        100        105        110        115        120
ATCCCAATCT CGCGAGGGCA AGCAGCAGGG TCTGCGGCGG CGGCGGCGGC CGCGCTTCCG

       125        130        135        140        145        150        155        160        165        170
GCTCCCCTTC CCATTGGCCT CCACG ATG GCG CTC CGC CTC AAC GAC GTC GCG
                            Met Ala Leu Arg Leu Asn Asp Val Ala>

175        180        185        190        195        200        205        210        215        220
CTC TGC CTC TCC CCG CCG CTC GCC GCC CGC CGC CGC CGC CGC AGC AGC
Leu Cys Leu Ser Pro Pro Leu Ala Ala Arg Arg Arg Arg Arg Ser Ser>

   225        230        235        240        245        250        255        260        265
GGC AGG TTC GTC GCC GTC GCC TCC ATG ACG TCC GCC GTC TCC ACC AAG
Gly Arg Phe Val Ala Val Ala Ser Met Thr Ser Ala Val Ser Thr Lys>

270        275        280        285        290        295        300        305        310        315
GTC GAG AAT AAG AAG CCA TTT GCT CCT CCA AGG GAG GTA CAT GTC CAG
Val Glu Asn Lys Lys Pro Phe Ala Pro Pro Arg Glu Val His Val Gln>

  320        325        330        335        340        345        350        355        360
GTT ACA CAT TCA ATG CCA CCT CAC AAG ATT GAA ATT TTC AAG TCG CTT
Val Thr His Ser Met Pro Pro His Lys Ile Glu Ile Phe Lys Ser Leu>

365        370        375        380        385        390        395        400        405        410
GAT GAT TGG GCT AGA GAT AAT ATC TTG ACG CAT CTC AAG CCA GTC GAG
Asp Asp Trp Ala Arg Asp Asn Ile Leu Thr His Leu Lys Pro Val Glu>

415        420        425        430        435        440        445        450        455        460
AAG TGT TGG CAG CCA CAG GAT TTC CTC CCG GAC CCA GCA TCT GAA GGA
Lys Cys Trp Gln Pro Gln Asp Phe Leu Pro Asp Pro Ala Ser Glu Gly>

   465        470        475        480        485        490        495        500        505
TTT CAT GAT GAA GTT AAG GAG CTC AGA GAA CGT GCC AAG GAA ATC CCT
Phe His Asp Glu Val Lys Glu Leu Arg Glu Arg Ala Lys Glu Ile Pro>
```

Figure 10

2

```
510       515    520       525       530    535       540       545    550       555
 *                *                   *                  *                 *
GAT GAT TAT TTT GTT TGT TTG GTG GGA GAC ATG ATT ACC GAG GAA GCT
Asp Asp Tyr Phe Val Cys Leu Val Gly Asp Met Ile Thr Glu Glu Ala>

   560    565       570       575    580       585       590    595       600
    *                *                 *                   *                 *
CTA CCA ACA TAC CAG ACT ATG CTT AAC ACC CTC GAC GGT GTC AGA GAT
Leu Pro Thr Tyr Gln Thr Met Leu Asn Thr Leu Asp Gly Val Arg Asp>

605    610       615       620    625       630       635    640       645       650
        *                   *                  *                  *                  *
GAG ACA GGT GCA AGC CCC ACT GCC TGG GCT GTT TGG ACG AGG GCA TGG
Glu Thr Gly Ala Ser Pro Thr Ala Trp Ala Val Trp Thr Arg Ala Trp>

655       660       665    670       675       680    685       690       695    700
           *                 *                   *                  *                 *
ACT GCT GAG GAG AAC AGG CAT GGT GAT CTG CTC AAC AAG TAT ATG TAC
Thr Ala Glu Glu Asn Arg His Gly Asp Leu Leu Asn Lys Tyr Met Tyr>

   705       710    715       720       725    730       735       740    745
              *                 *                  *                  *
CTC ACT GGG AGG GTG GAT ATG AGG CAG ATT GAG AAG ACA ATT CAG TAT
Leu Thr Gly Arg Val Asp Met Arg Gln Ile Glu Lys Thr Ile Gln Tyr>

750       755    760       765       770    775       780       785    790       795
 *                *                   *                   *                 *
CTT ATT GGC TCT GGA ATG GAT CCT AGG ACT GAG AAT AAT CCT TAT CTT
Leu Ile Gly Ser Gly Met Asp Pro Arg Thr Glu Asn Asn Pro Tyr Leu>

   800    805       810       815    820       825       830    835       840
    *                *                  *                   *                 *
GGT TTC ATC TAC ACC TCC TTC CAA GAG CGG GCG ACC TTC ATC TCA CAC
Gly Phe Ile Tyr Thr Ser Phe Gln Glu Arg Ala Thr Phe Ile Ser His>

845    850       855       860    865       870       875    880       885       890
        *                   *                  *                  *                  *
GGG AAC ACT GCT CGT CAC GCC AAG GAC TTT GGC GAC TTA AAG CTT GCA
Gly Asn Thr Ala Arg His Ala Lys Asp Phe Gly Asp Leu Lys Leu Ala>

895       900       905    910       915       920    925       930       935    940
           *                 *                   *                  *                 *
CAA ATC TGC GGC ATC ATC GCC TCA GAT GAG AAG CGA CAT GAA ACT GCG
Gln Ile Cys Gly Ile Ile Ala Ser Asp Glu Lys Arg His Glu Thr Ala>

   945       950    955       960       965    970       975       980    985
              *                 *                  *                  *
TAC ACC AAG ATC GTG GAG AAG CTG TTT GAG ATC GAC CCT GAT GGT ACC
Tyr Thr Lys Ile Val Glu Lys Leu Phe Glu Ile Asp Pro Asp Gly Thr>

990       995 1000      1005      1010 1015      1020      1025 1030      1035
 *                *                   *                   *                 *
GTG GTC GCT CTG GCT GAC ATG ATG AGG AAG AAG ATC TCA ATG CCT GCC
Val Val Ala Leu Ala Asp Met Met Arg Lys Lys Ile Ser Met Pro Ala>
```

3

```
 1040  1045   1050   1055  1060   1065   1070  1075   1080
    *            *            *            *            *
CAC CTG ATG TTT GAC GGG CAG GAC GAC AAG CTG TTC GAG CAC TTC TCC
His Leu Met Phe Asp Gly Gln Asp Asp Lys Leu Phe Glu His Phe Ser>

1085  1090   1095   1100  1105   1110   1115  1120   1125   1130
         *            *            *            *            *
ATG GTC GCG CAG AGG CTT GGC GTT TAC ACC GCC AGG GAC TAC GCC GAC
Met Val Ala Gln Arg Leu Gly Val Tyr Thr Ala Arg Asp Tyr Ala Asp>

 1135   1140   1145  1150   1155   1160  1165   1170   1175  1180
           *            *            *            *            *
ATC CTC GAG TTC CTC GTC GAC AGG TGG AAG GTG GCG AGC CTG ACT GGT
Ile Leu Glu Phe Leu Val Asp Arg Trp Lys Val Ala Ser Leu Thr Gly>

   1185   1190  1195   1200   1205  1210   1215   1220  1225
             *            *            *            *
CTG TCG GGT GAA GGG AAC AAG GCG CAG GAC TAC CTT TGC ACC CTT GCT
Leu Ser Gly Glu Gly Asn Lys Ala Gln Asp Tyr Leu Cys Thr Leu Ala>

 1230   1235  1240   1245   1250  1255   1260   1265  1270   1275
    *            *            *            *            *
TCA AGA ATC AGG AGG CTG GAG GAG AGG GCC CAG AGC AGA GCC AAG AAA
Ser Arg Ile Arg Arg Leu Glu Glu Arg Ala Gln Ser Arg Ala Lys Lys>

  1280  1285   1290   1295  1300   1305   1310  1315   1320
     *            *            *            *            *
GCC GGC ACG CTG CCT TTC AGC TGG GTA TAC GGT AGG GAC GTC CAA CTG
Ala Gly Thr Leu Pro Phe Ser Trp Val Tyr Gly Arg Asp Val Gln Leu>

1325  1330  1335 1340  1345 1350  1355 1360  1365 1370  1375 1380
         *          *          *          *          *          *
TGA GAT CGGAAACCTG CTGCGGTCTG CTTAGACAAG ACCTGCTGTG TCTGCGTTAC
***>

  1385 1390  1395 1400  1405 1410  1415 1420  1425 1430  1435 1440
         *          *          *          *          *          *
ATAGGTCTCC AGGTTTTGAT CAAATGGTCC CGTGTCGTCT TATAGAGCGA TAGGAGAACG

  1445 1450  1455 1460  1465 1470  1475 1480  1485 1490  1495 1500
         *          *          *          *          *          *
TGTTGGTCTG TGGTGTAGCT TTGTTTTTAT TTTGTATTTT TCTGCTTTGA TGTACAACCT

  1505 1510  1515 1520  1525 1530  1535 1540  1545 1550  1555 1560
         *          *          *          *          *          *
GTGGCCGCAT GAACTGGGGC GTGGAGATGG GAGCGACCAT GCCGTACTTT GTCTGTCGCT

  1565 1570  1575 1580  1585 1590  1595 1600  1605 1610  1615 1620
         *          *          *          *          *          *
GGCGGTGTGT TTCGGTATGT TATTTGAGTT GCTCAGATCT GTTAAAAAAA AAAAAAAAAA

A
```

Figure 11: Fatty Acid Biosynthesis and Modification

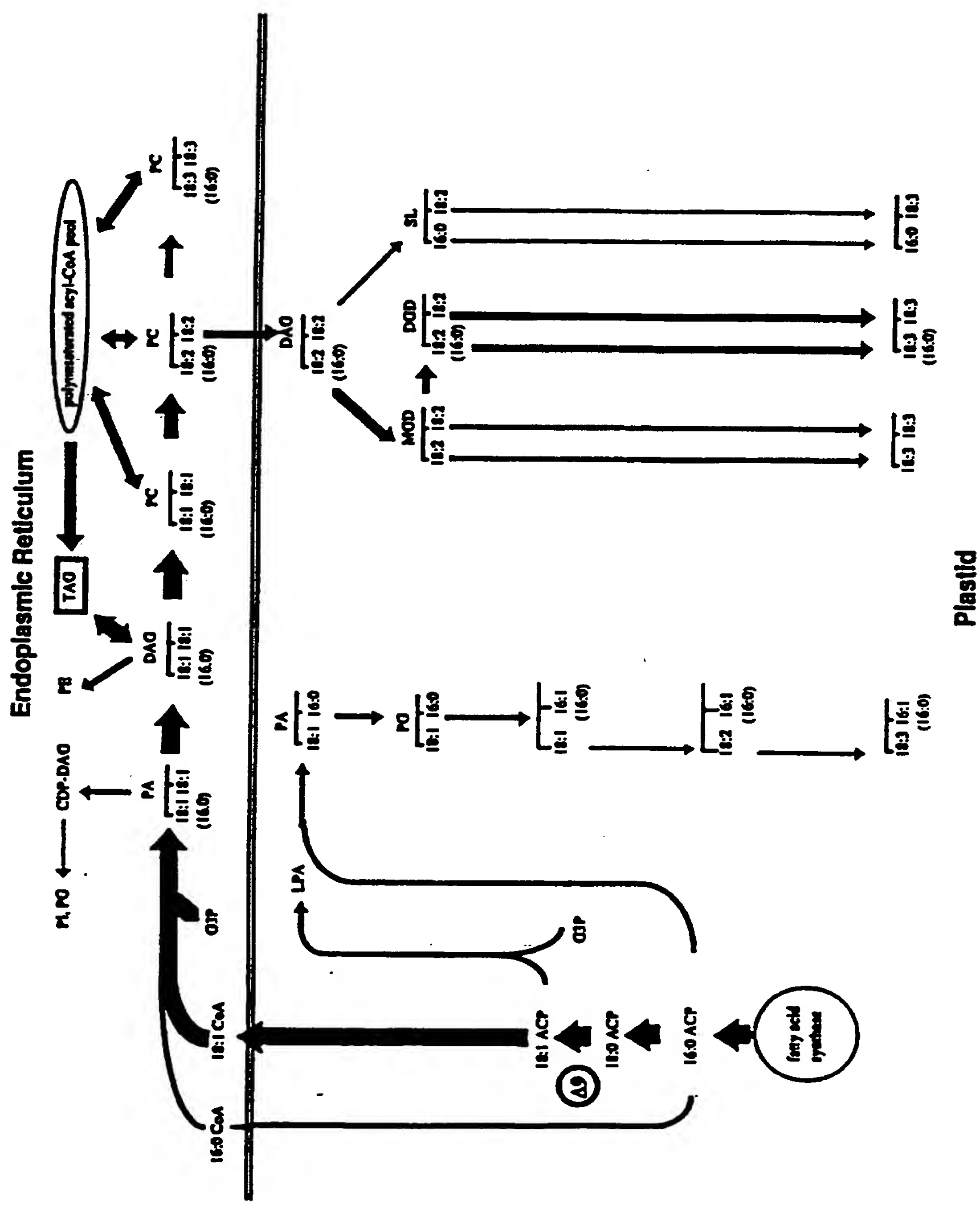
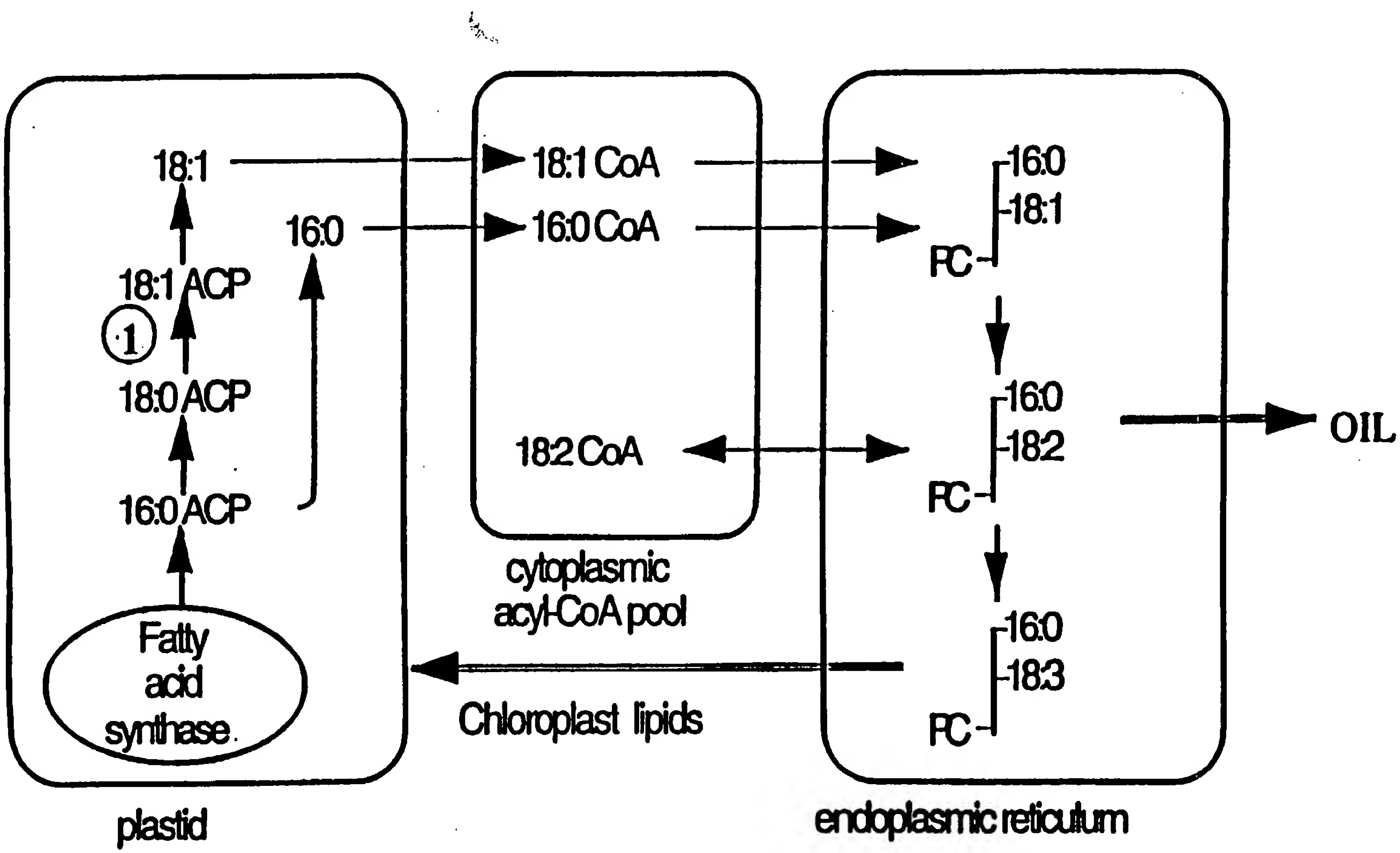
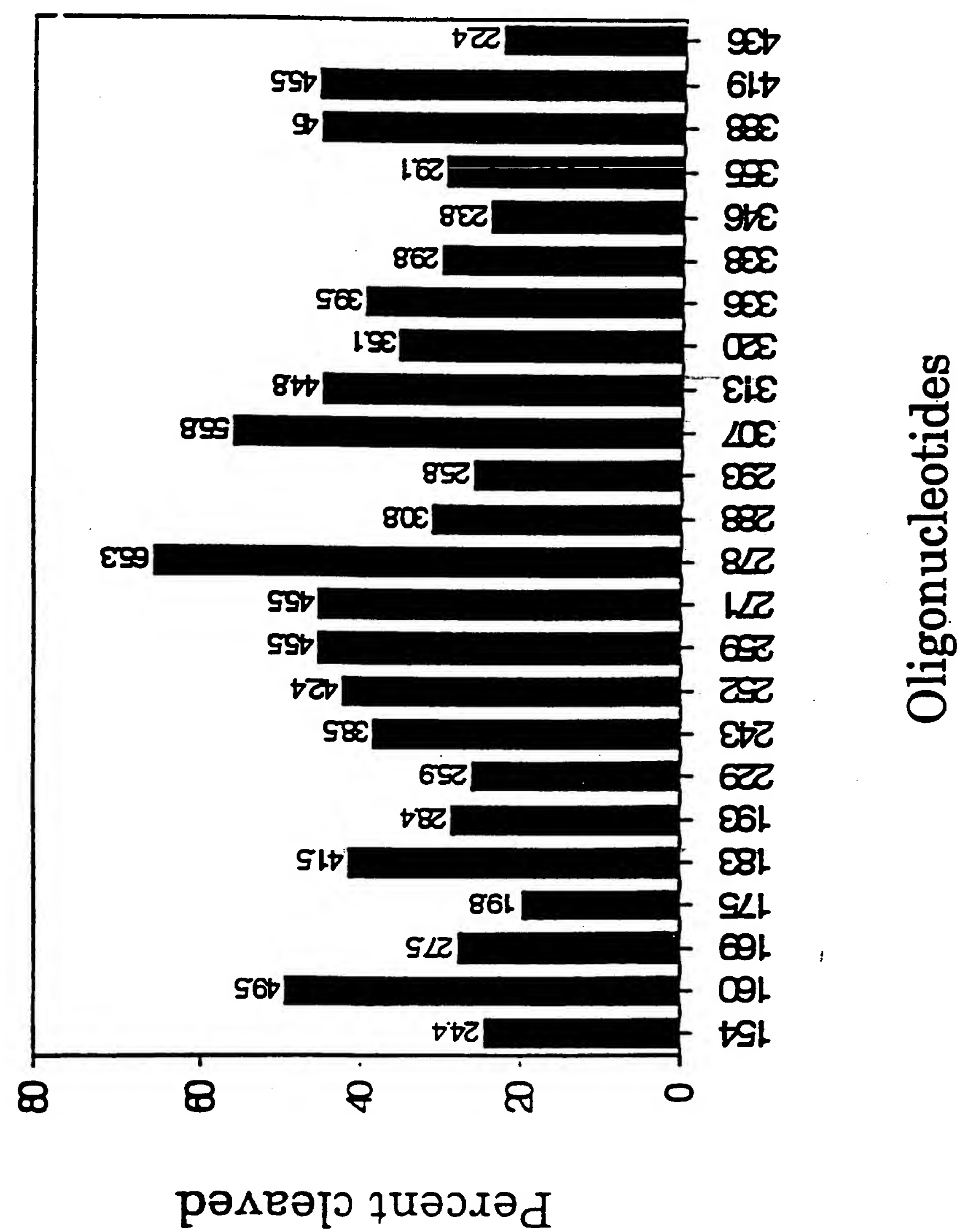


Figure 12: Plant Fatty Acid Biosynthesis



1. Δ9 desaturase

Figure 13: RNase H Accessibility of Δ9-Desaturase RNA



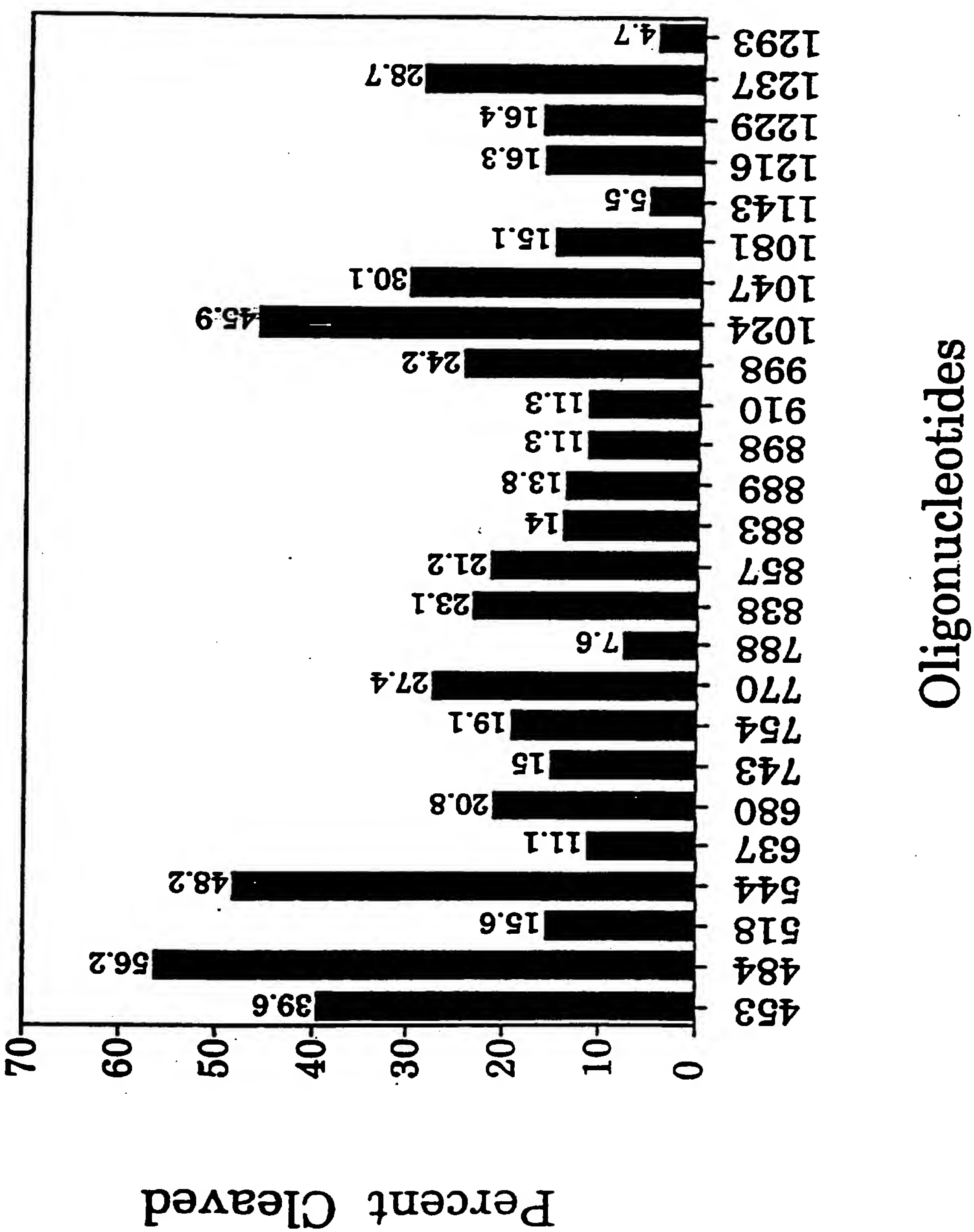
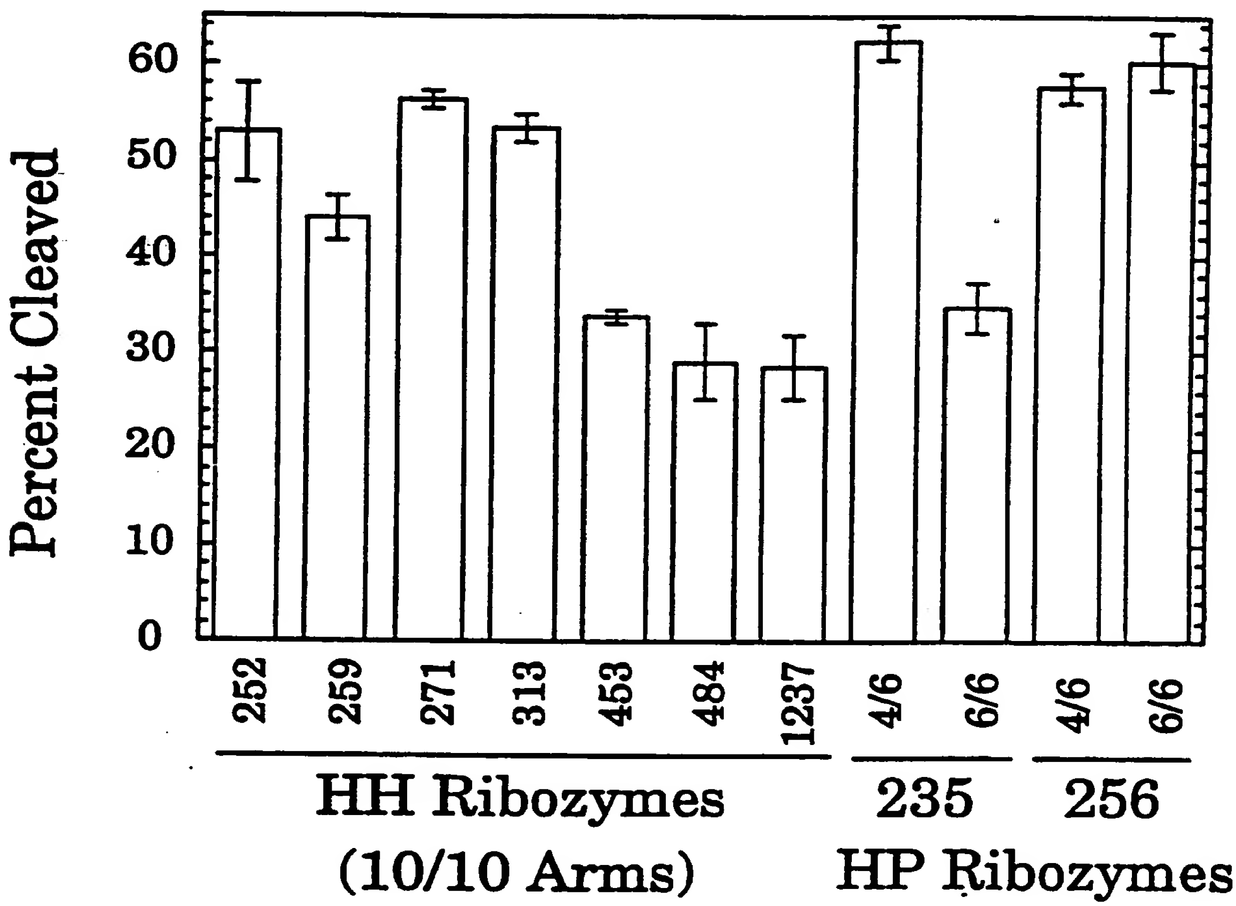


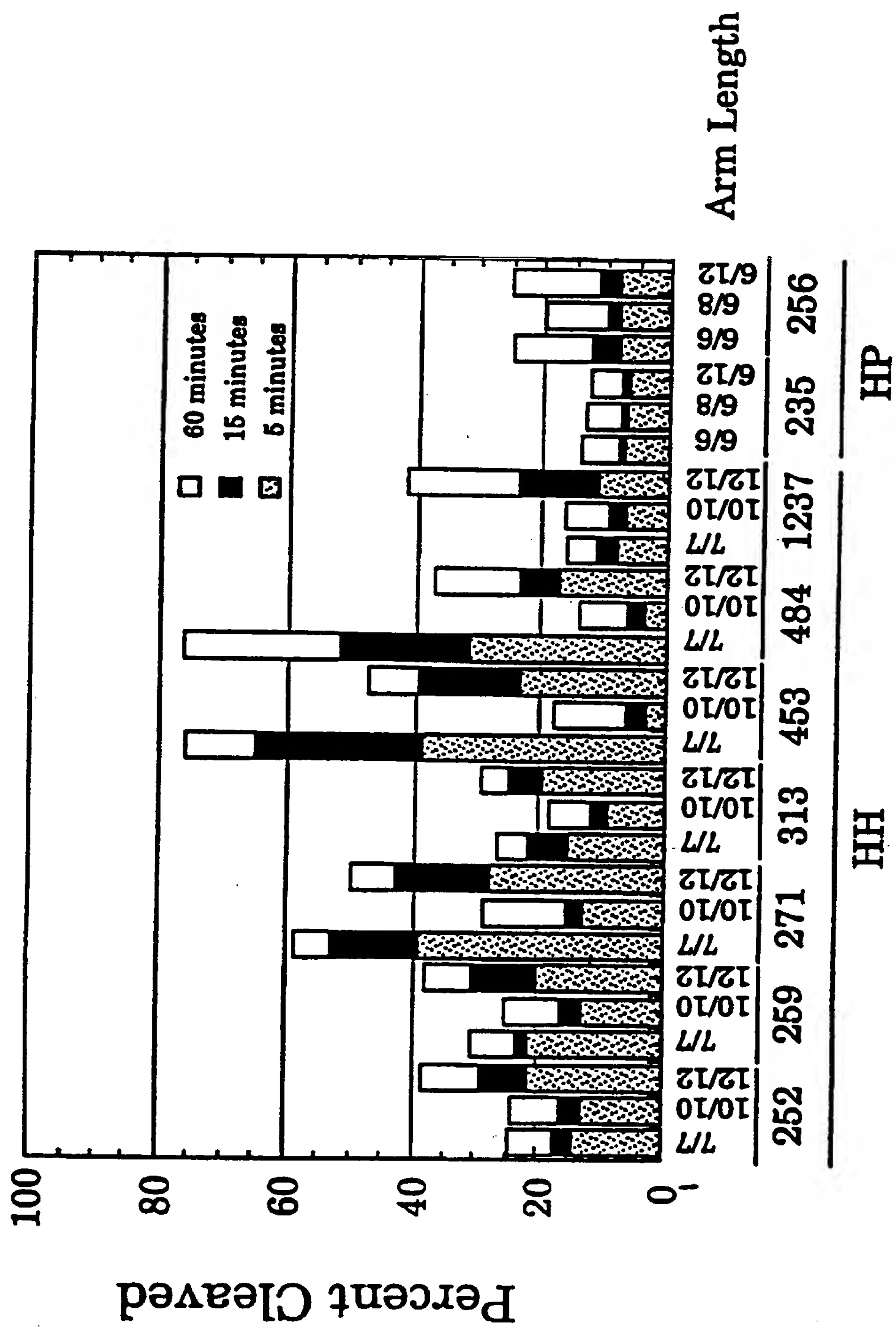
Figure 14: RNase H Accessibility of Δ9-Desaturase RNA

Figure 15: Cleavage of Δ-9 Desaturase RNA by Ribozymes *in vitro*



[Ribozyme] = 1μM [Long Substrate] = ~10 nM

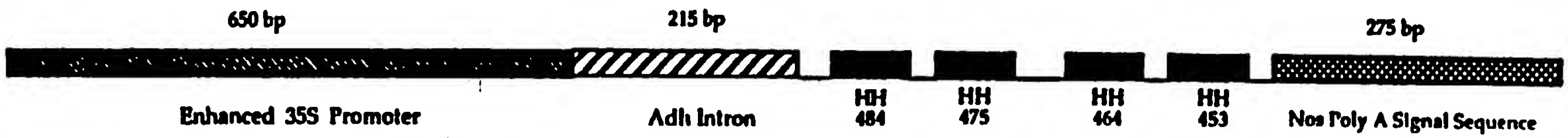
Figure 16: Effect of Arm-Length Variation on Ribozyme Activity



[Ribozyme] = 1μM [Long Substrate] = ~10 nM

Figure 17: Delta-9 Desaturase Multimer Ribozyme Construct

CaMV 35S Enhanced promoter/Adh intron/ 4 HH Ribozymes/Nos poly A



Delta-9 Desaturase Multimer Ribozyme Transcript

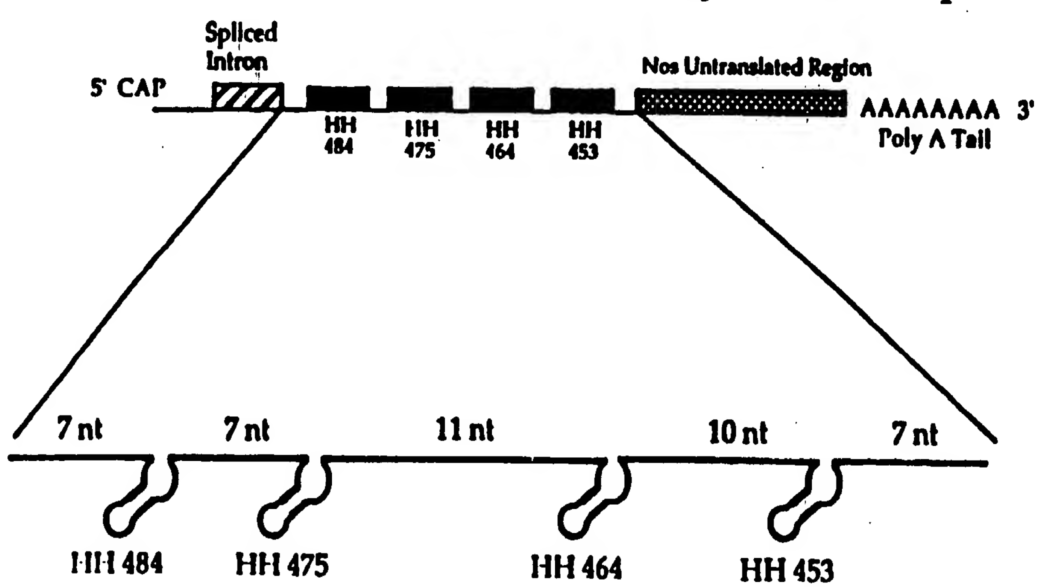
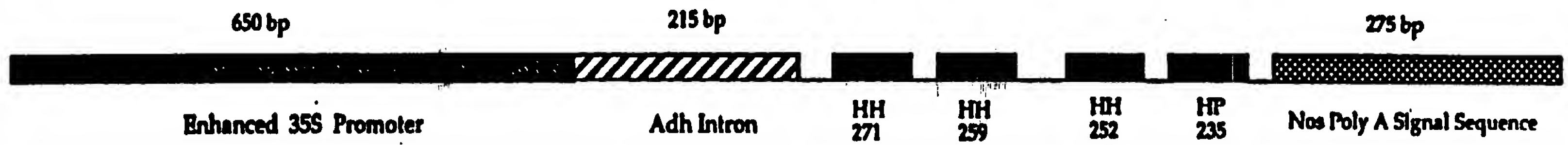


Figure 18: Delta-9 Desaturase Multimer Ribozyme Construct

Multimer Constuct

CaMV 35S Enhanced promoter/Adh intron/ 4 Ribozymes/Nos poly A



Delta-9 Desaturase Multimer Ribozyme Transcript

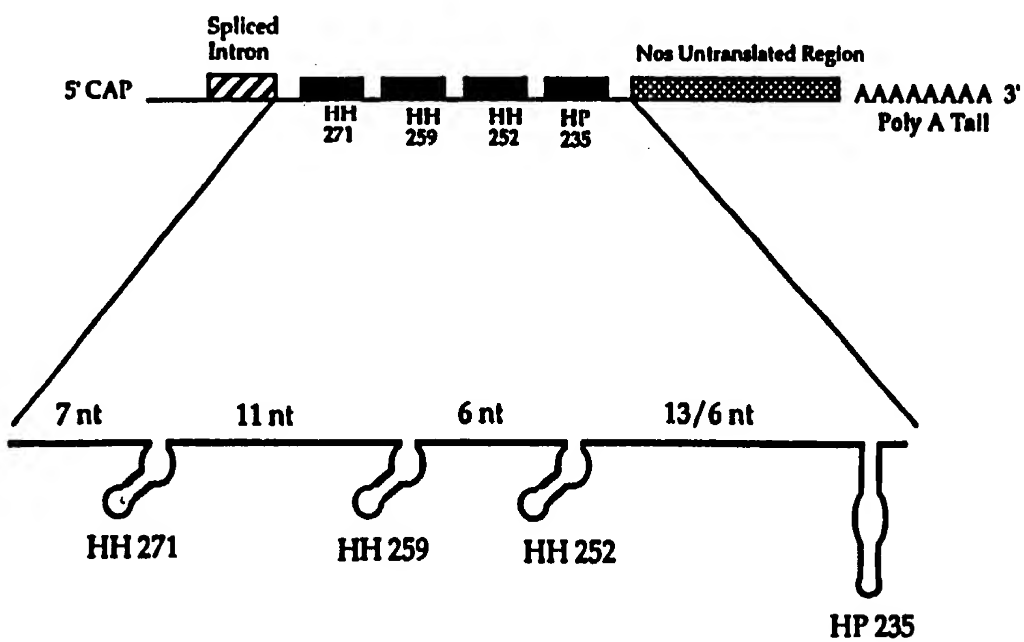
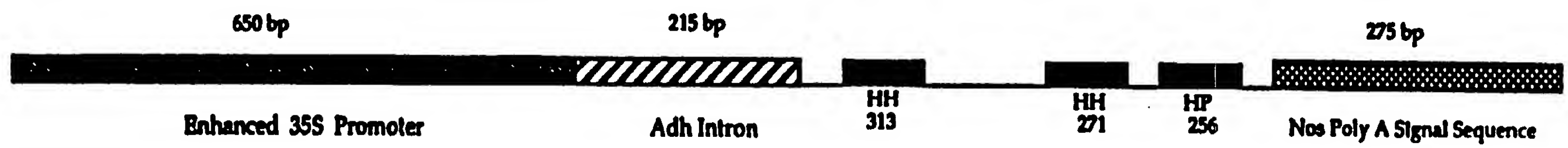


Figure 19: Delta-9 Desaturase Multimer Ribozyme

Multimer Constuct

CaMV 35S Enhanced promoter/Adh intron/ 3 Ribozymes/Nos poly A



Delta-9 Desaturase Multimer Ribozyme Transcript

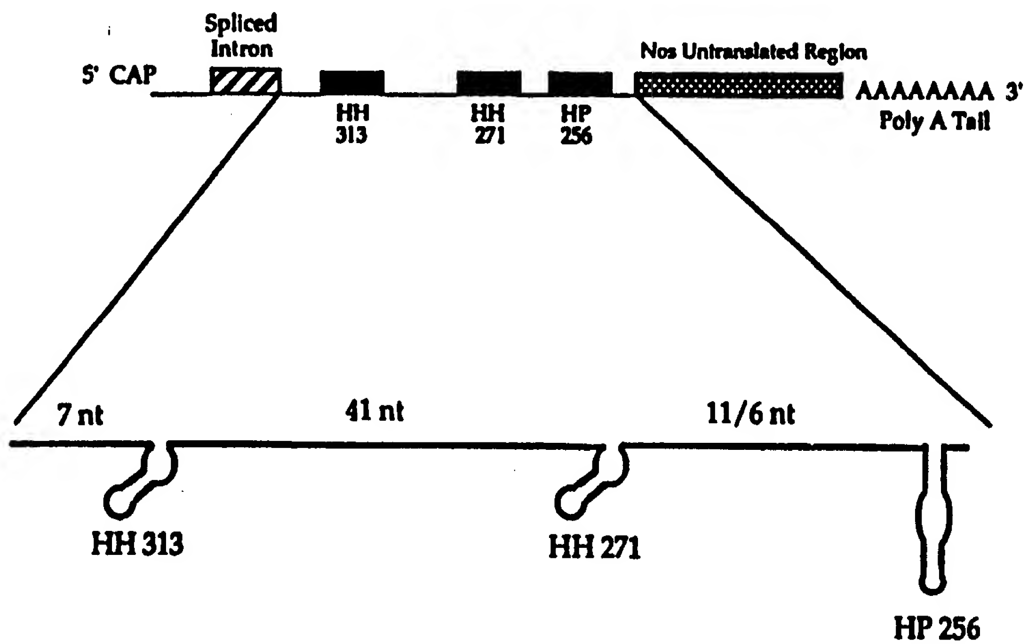
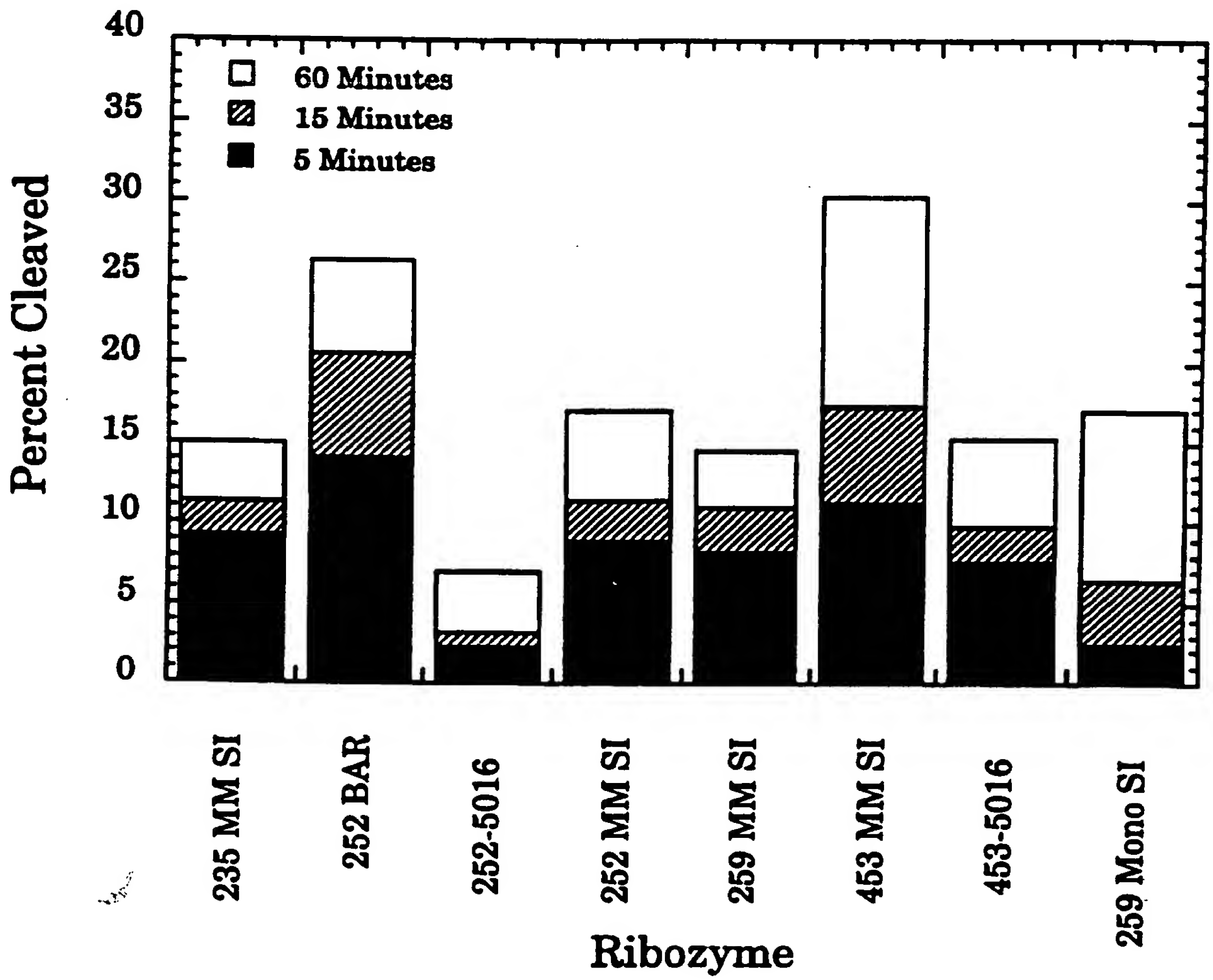


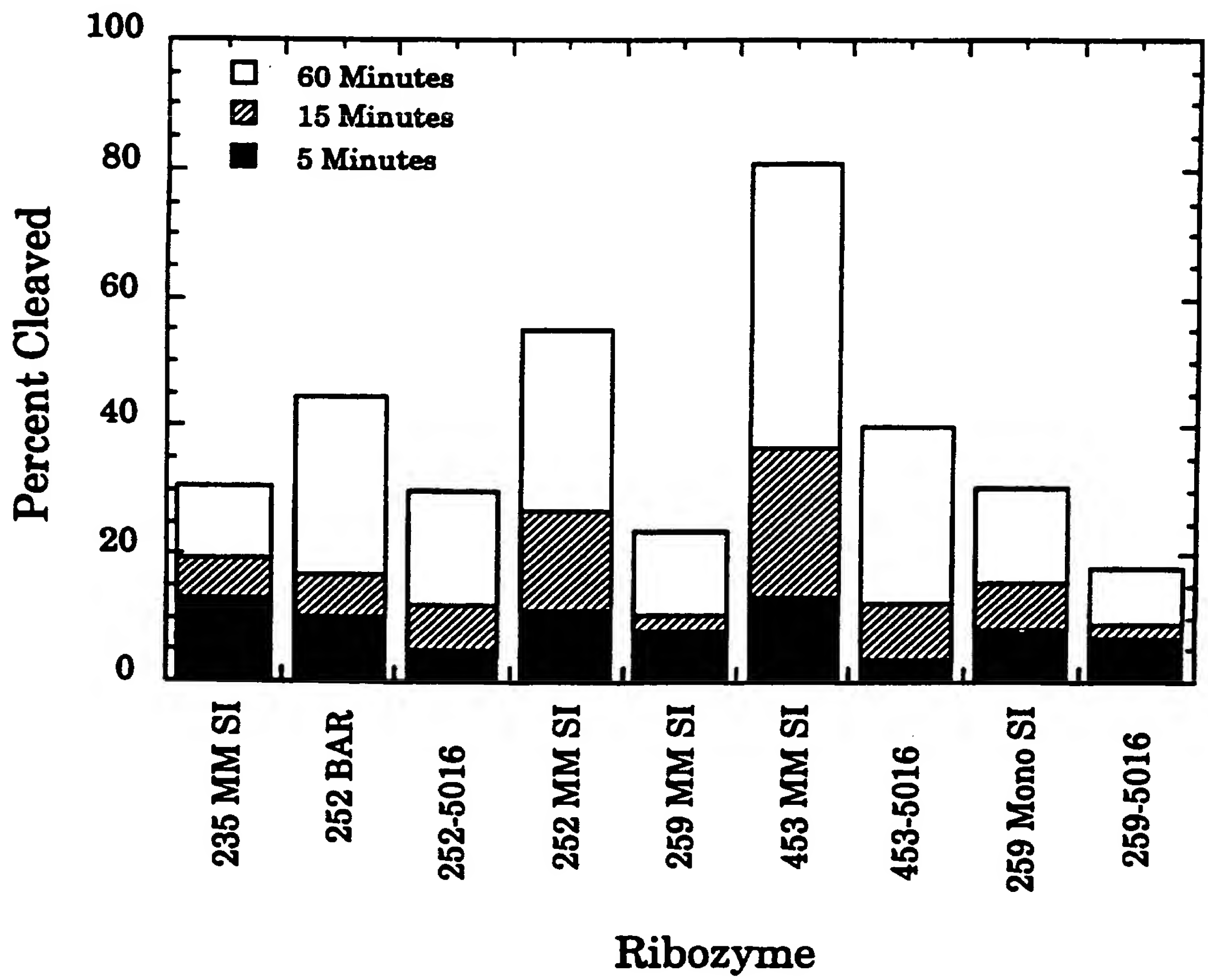
Fig 20: Cleavage of Delta-9 RNA by Ribozymes



MM= Multimer Rz
SI= Spliced Intron Transcript
BAR= RZ at 3' end ORF of BAR
5016= 5' minimal leader, 3' intron

[Long Substrate] = 10 nM; [Ribozyme] = 1 µM; Temperature = 26°C

Fig 21: Cleavage of Delta-9 RNA by Ribozymes

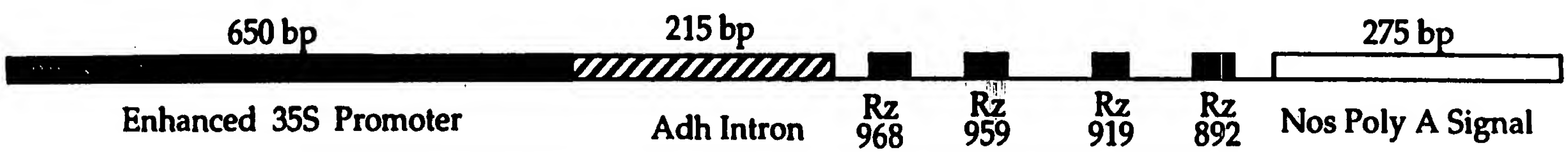


MM= Multimer Rz
SI= Spliced Intron Transcript
BAR= RZ at 3' end ORF of BAR
5016= 5' minimal leader, 3' intron

[Long Substrate] = 10 nM; [Ribozyme] = 1 μM; Temperature = 37°C

Figure 22: GBSS Multimer Ribozyme Construct

CaMV 35S Enhanced promoter/Adh intron/ 4 Rzs imbedded in antisense sequence/Nos poly A



Multimer Ribozyme Transcript

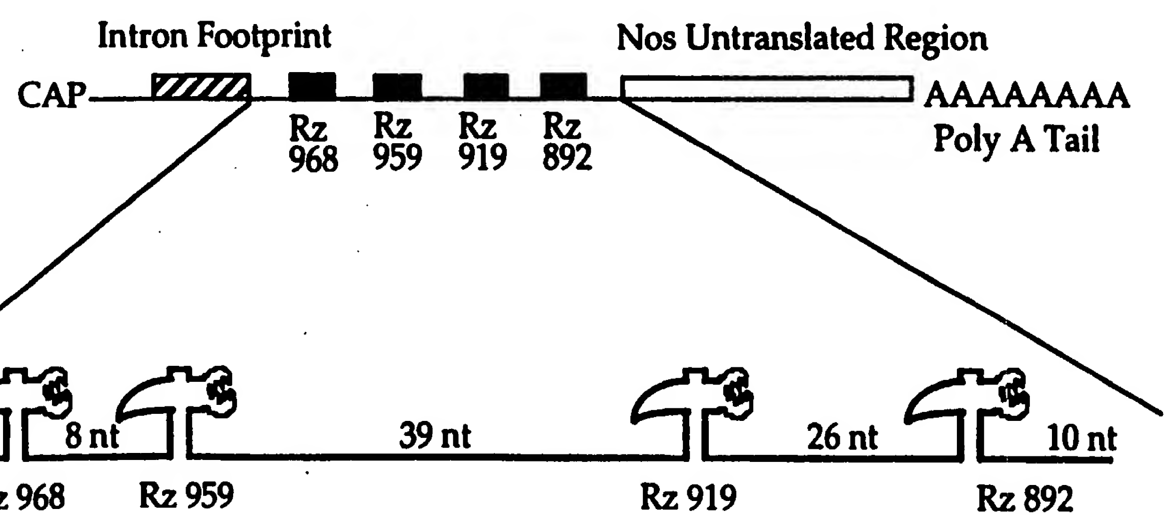


Figure 23: Delta-9 Desaturase Multimer Ribozyme

Multimer Constuct

CaMV 35S Enhanced promoter/Adh intron/ 4 Rzs imbedded in antisense sequence/Nos poly A

Multimer Ribozyme Transcript

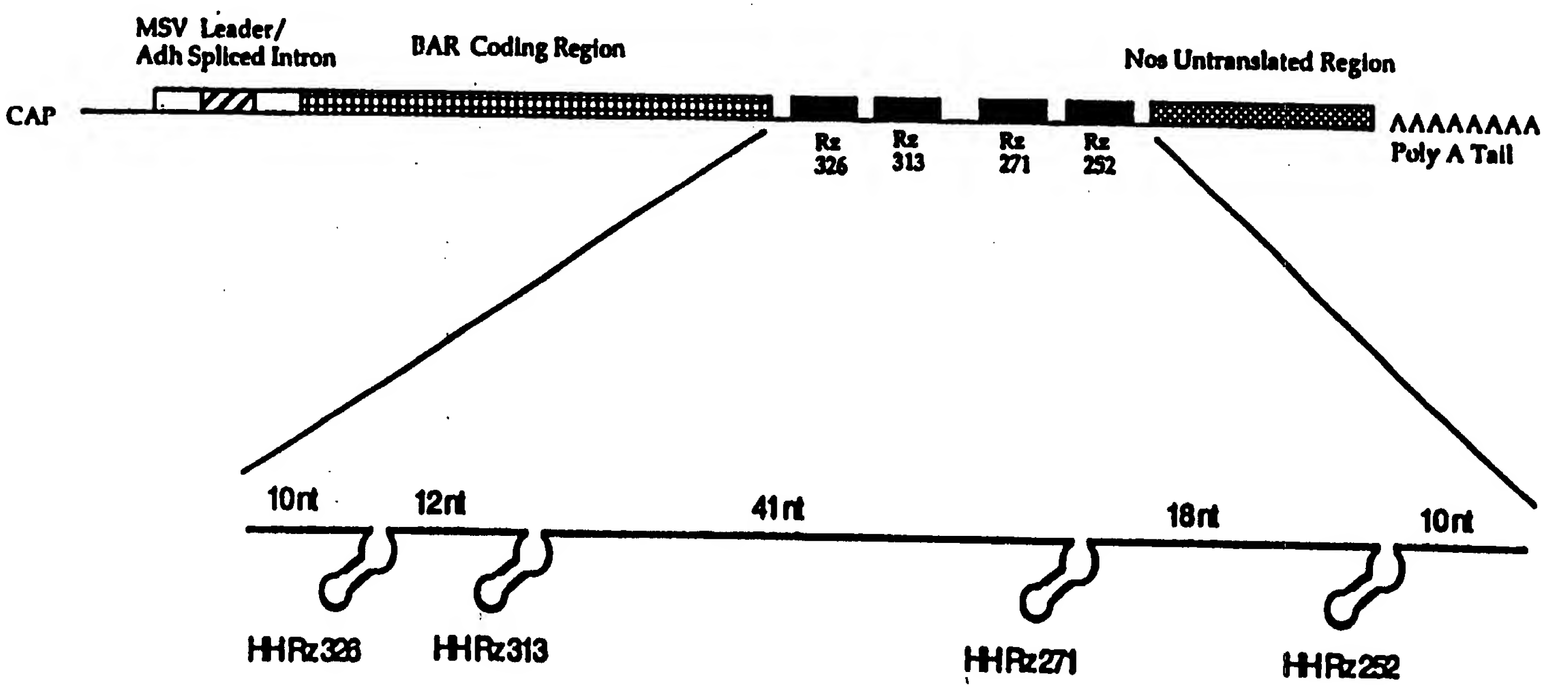


Figure 24: Cleavage of Delta-9 Desaturase RNA by Ribozymes

Ribozymes	Percent Cleaved
453 Multimer	*79.2*
453	47
464	≥ 1
475	20
484	33
252 Multimer	*55.2*
252	55
271	20
313	20
326	5
238 Multimer	*30.9*
238 HP	≥ 1
252	33
259	≥ 1
271	67
259 Multimer	*24*
259 HP	9
271	40
313	51

Ribozyme Mediated Reductions in GBSS mRNA

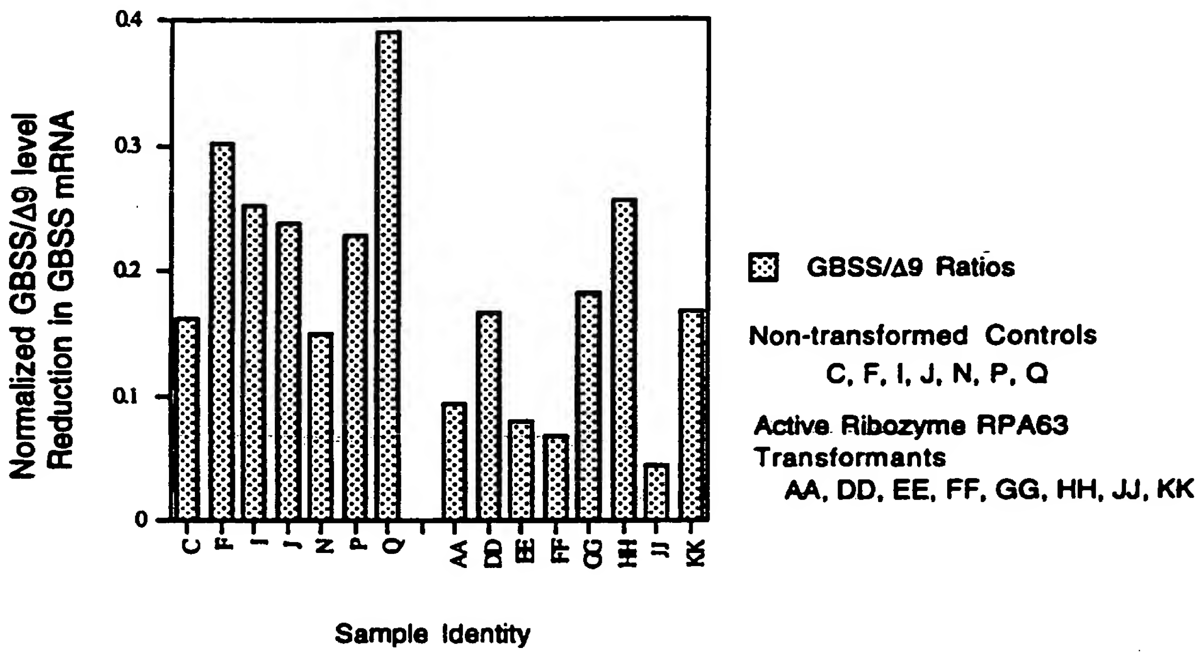


Figure 25

Figure Ribozyme Mediated Reductions in Δ9 mRNA in RPA85-06 Plants

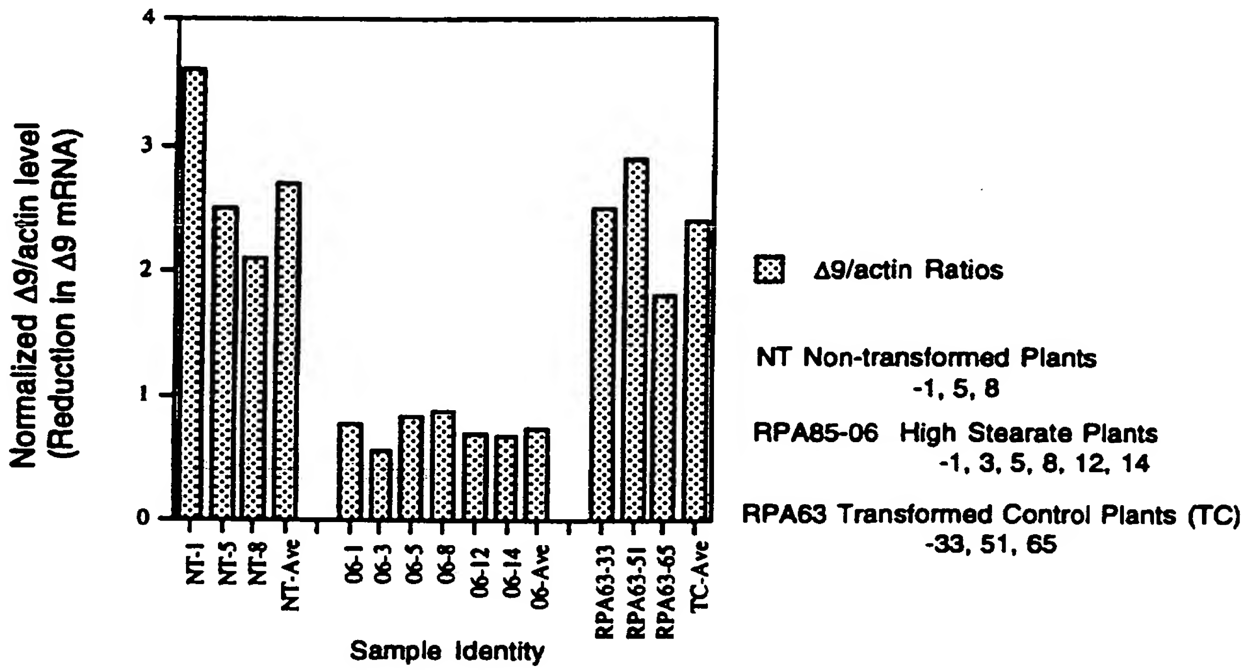


Figure 26

Ribozyme mediated reductions in Δ9 mRNA in RPA85-15 Plants

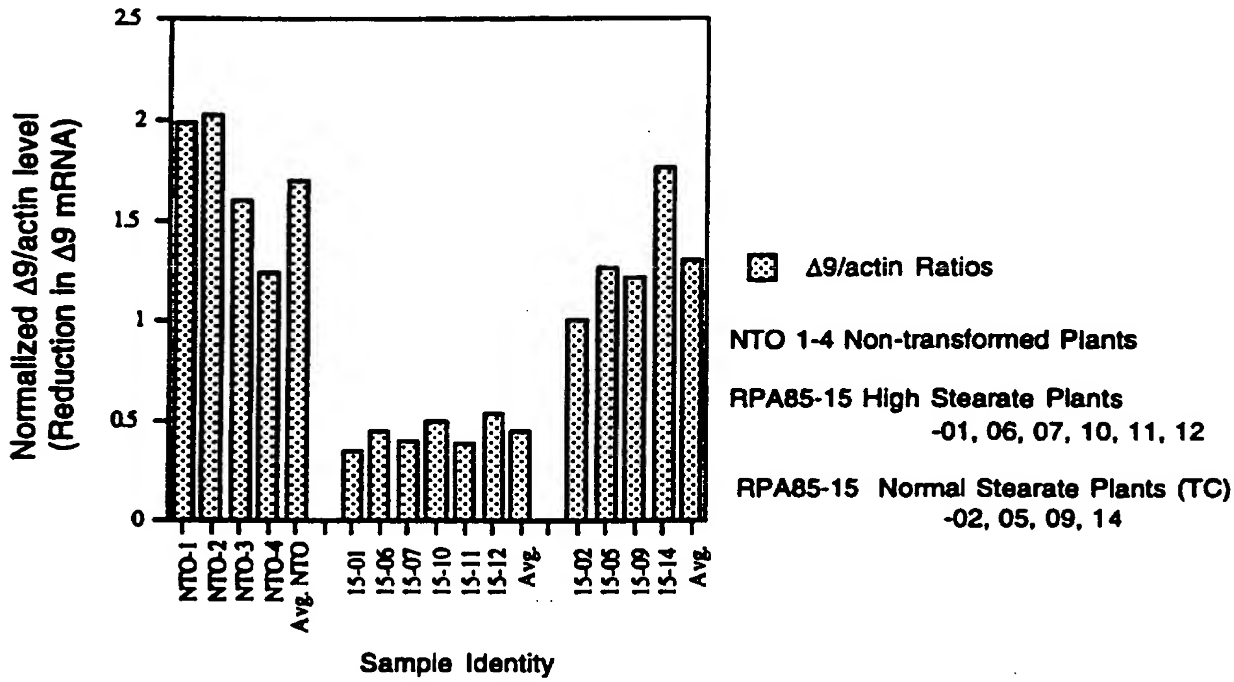


Figure 27

mRNA levels in Inactive Ribozyme Trangenic Line 113-06

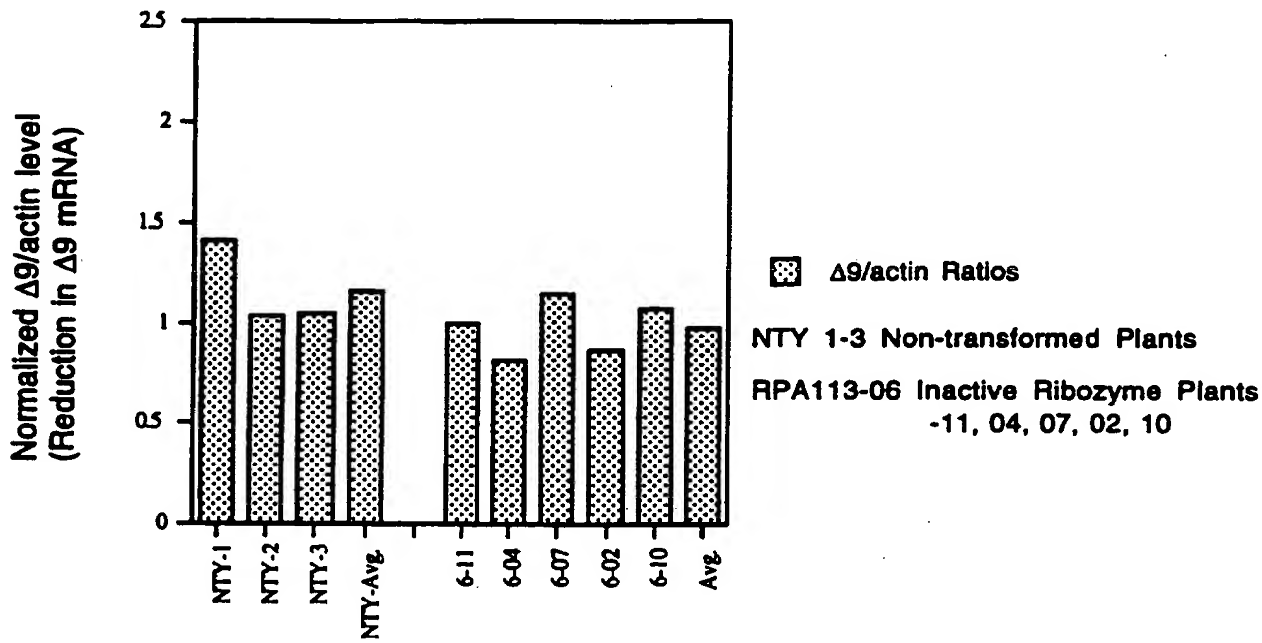


Figure 28

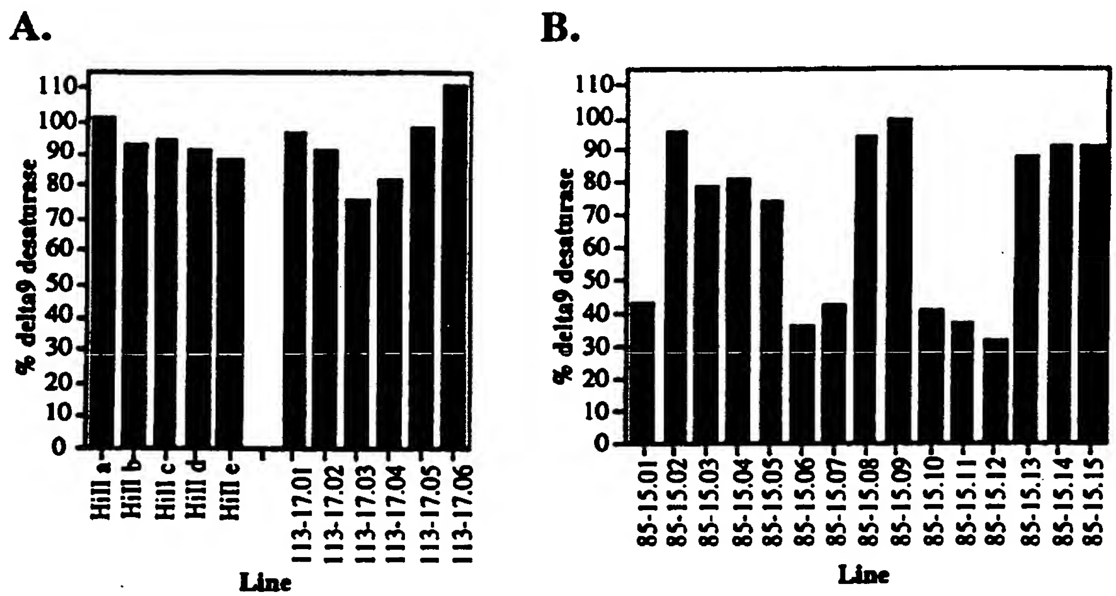

A.
% delta9 desaturase
0
10
20
30
40
50
60
70
80
90
100
110
Hill a
Hill b
Hill c
Hill d
Hill e
113-17.01
113-17.02
113-17.03
113-17.04
113-17.05
113-17.06
Line
B.
% delta9 desaturase
0
10
20
30
40
50
60
70
80
90
100
110
85-15.01
85-15.02
85-15.03
85-15.04
85-15.05
85-15.06
85-15.07
85-15.08
85-15.09
85-15.10
85-15.11
85-15.12
85-15.13
85-15.14
85-15.15
Line

Figure 29

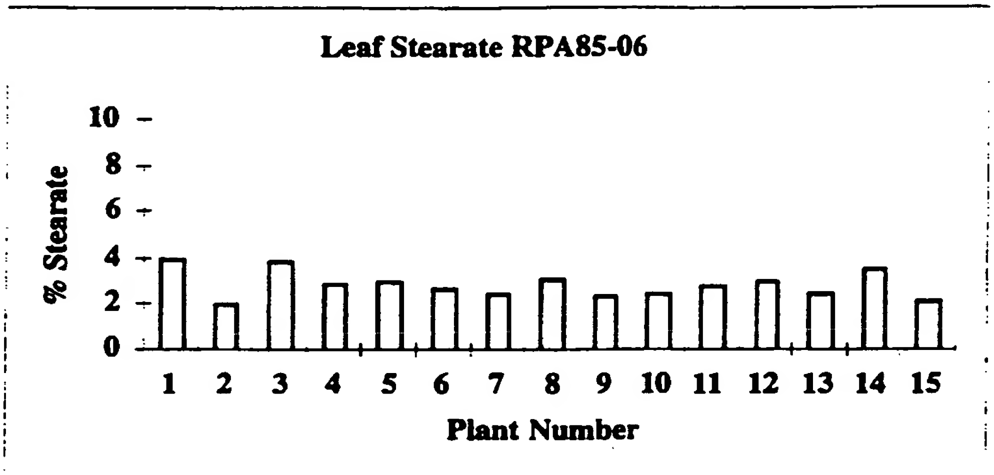

Leaf Stearate RPA85-06
% Stearate
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Plant Number

Figure 30

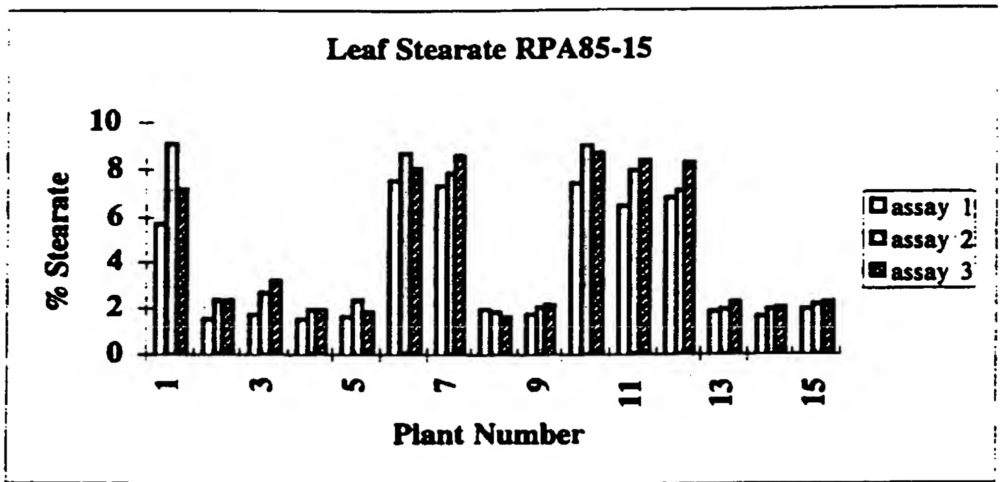

Leaf Stearate RPA85-15
% Stearate
10
8
6
4
2
0
1
3
5
7
9
11
13
15
Plant Number
assay 1
assay 2
assay 3

Figure 31

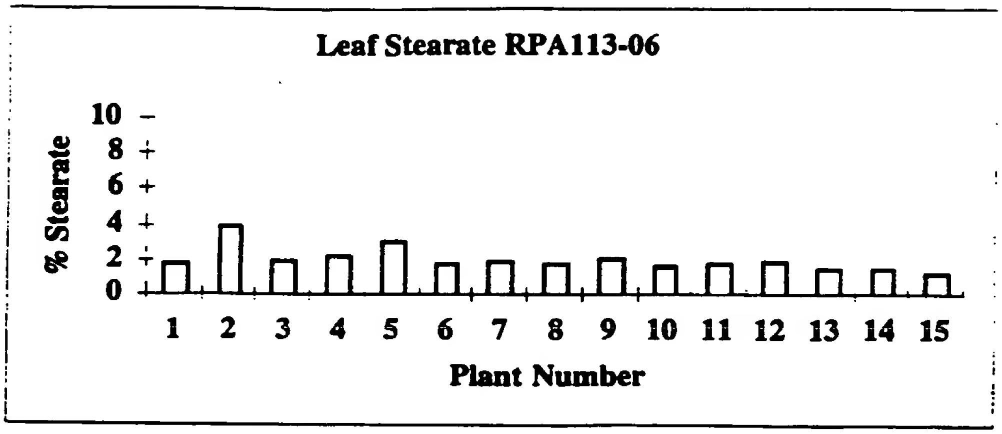

Leaf Stearate RPA113-06
% Stearate
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Plant Number

Figure 32

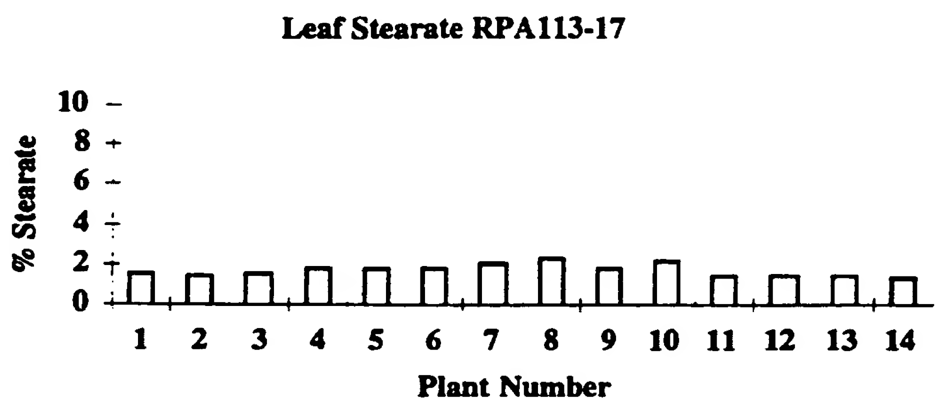

Leaf Stearate RPA113-17
% Stearate
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Plant Number

Figure 33

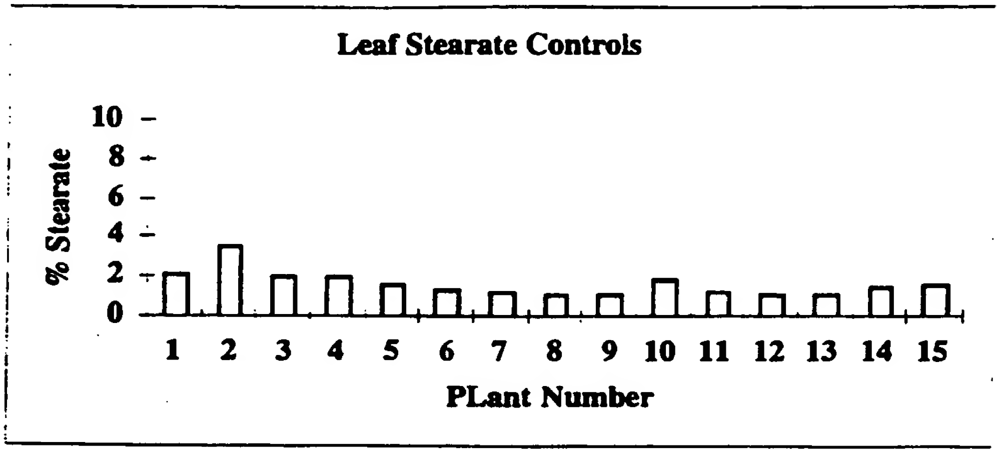

Leaf Stearate Controls
% Stearate
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PLant Number

Figure 34

Inheritance of High Stearate Phenotype

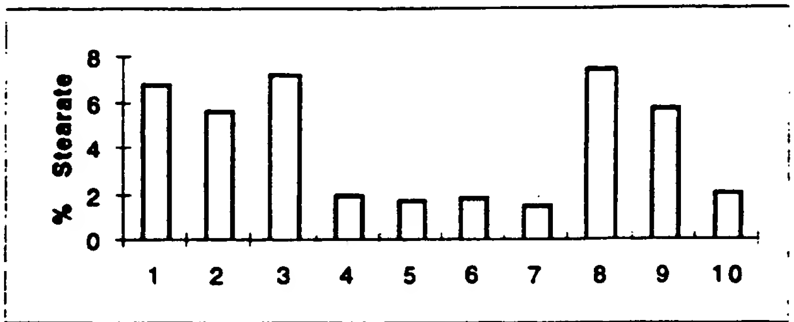


Figure 35

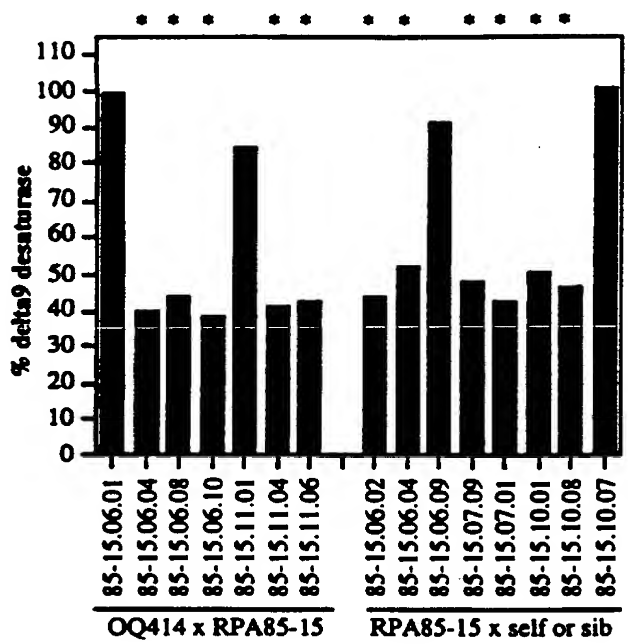

% delta9 desaturase
110
100
90
80
70
60
50
40
30
20
10
0
85-15.06.01
85-15.06.04
85-15.06.08
85-15.06.10
85-15.11.01
85-15.11.04
85-15.11.06
85-15.06.02
85-15.06.04
85-15.06.09
85-15.07.09
85-15.07.01
85-15.10.01
85-15.10.08
85-15.10.07
OQ414 x RPA85-15
RPA85-15 x self or sib

Figure 36

Antisense stearate phenotype

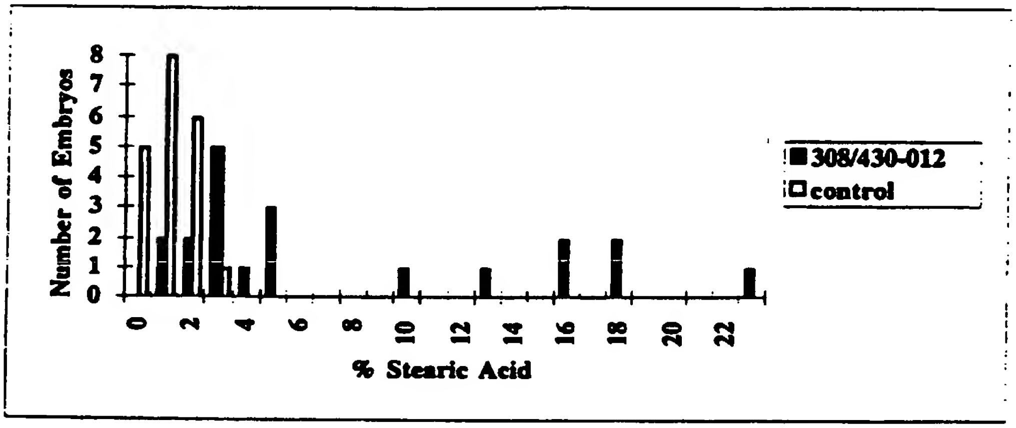


Figure 37

Antisense effect on stearate

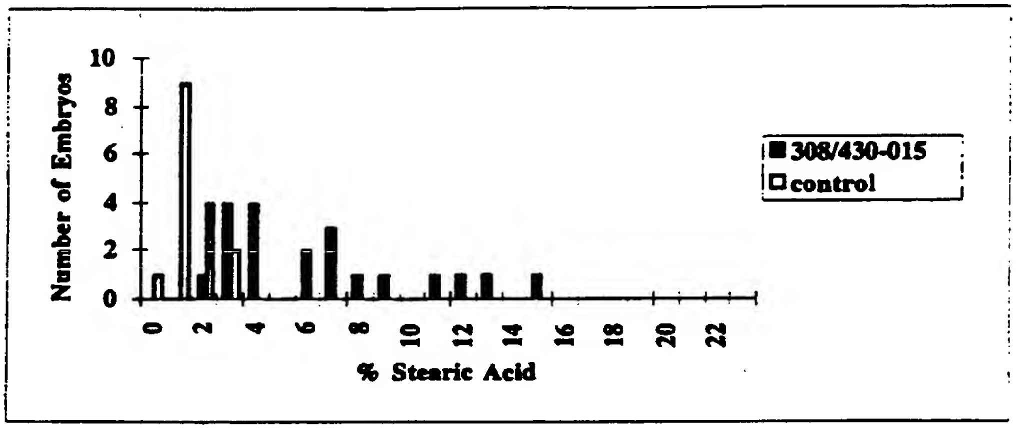


Figure 38

Antisense stearate effect

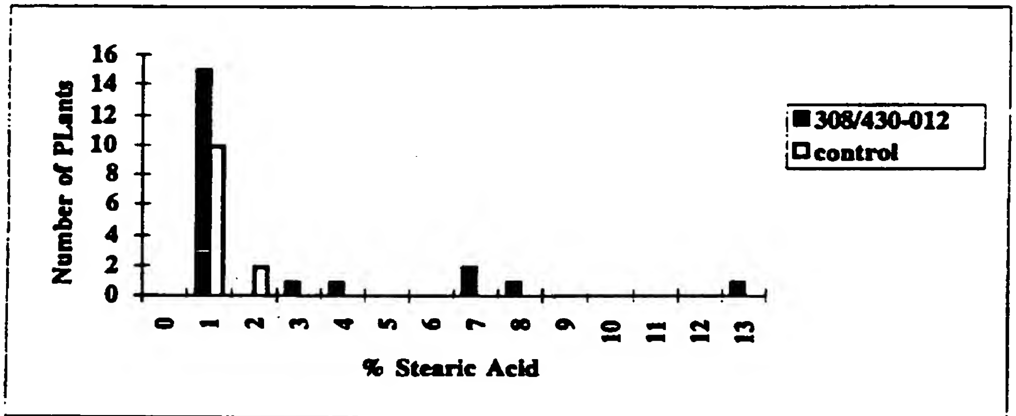


Figure 39

Antisense effect on amylose

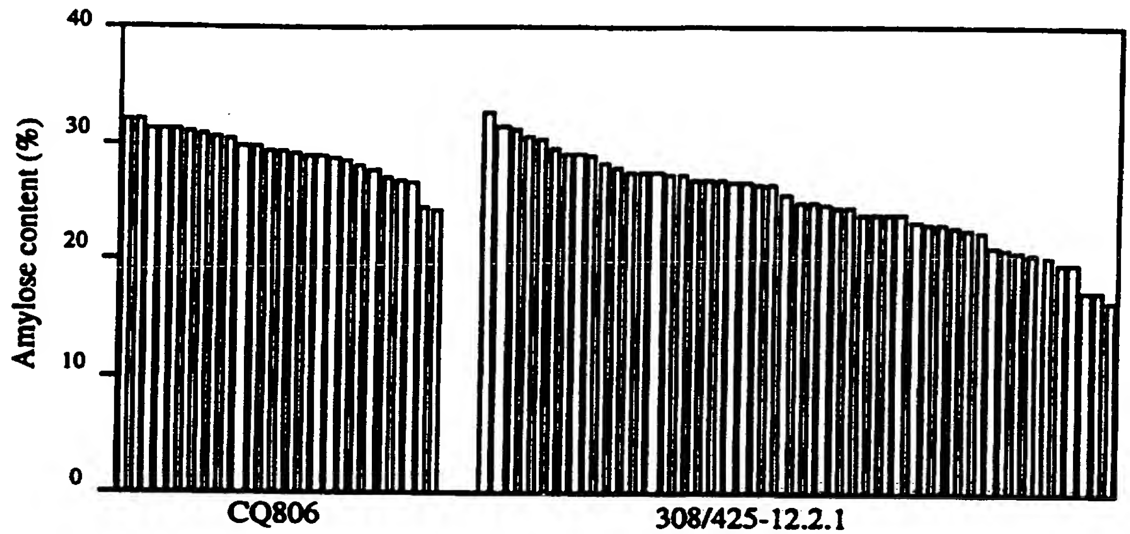


Figure 40

Ribozyme effect on GBSS activity

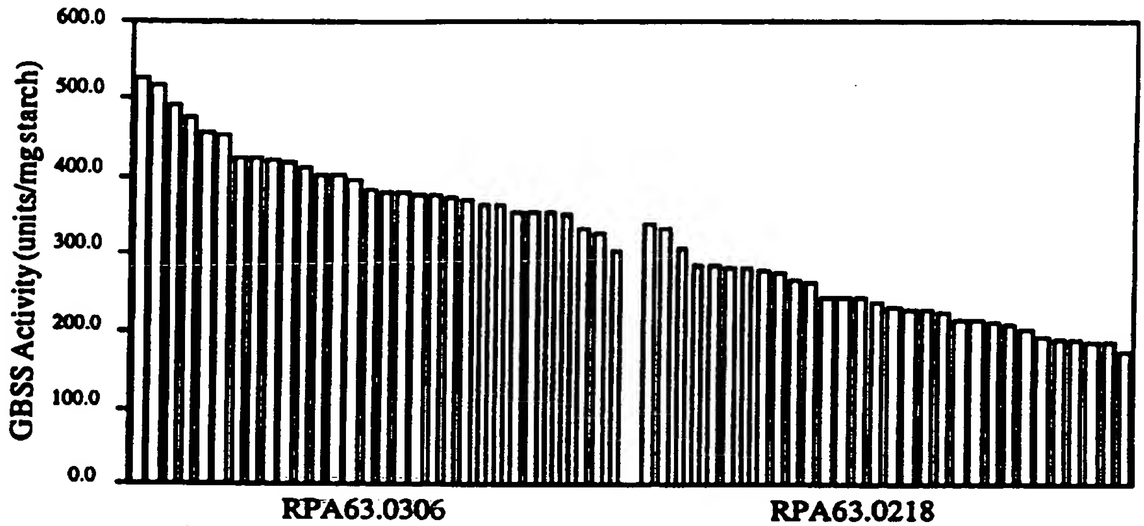
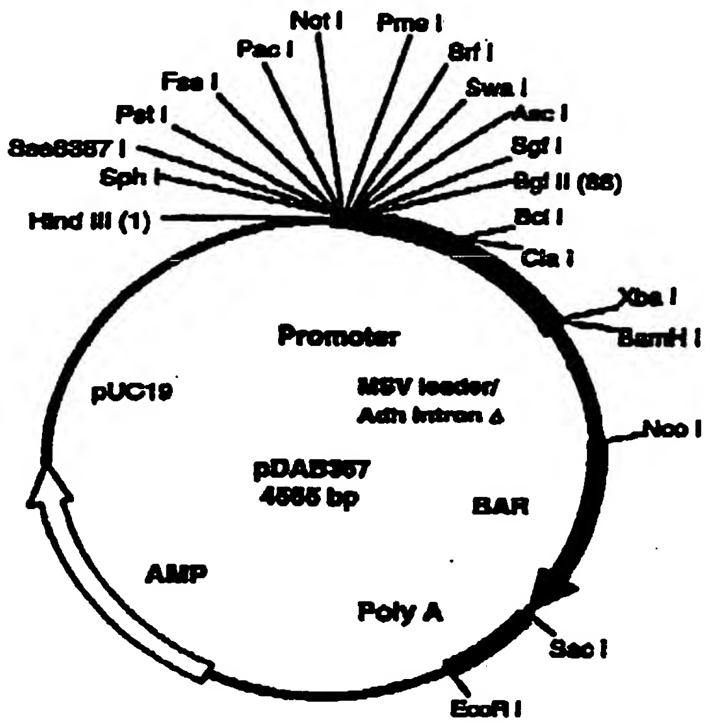


Figure 41

FIGURE 42



Sequence of nucleotides 1-91:

```
        Sph I  Pst I  Fse I
Hind III     Sse8387 I      Pac I    Not I    Pme I    Srf I    Swa I    Asc I    Sgf I        Bgl II
AAGCTTGCATGCCTGCAGGCCGGCCTTAATTAAGCGGCCGCGTTTAAACGCCCGGGCATTTAAATGGCGCGCCGCGATCGCTTGCAGATCT
TTCGAACGTACGGACGTCCGGCCGGAATTAATTCGCCGGCGCAAATTTGCGGGCCCGTAAATTTACCGCGCGGCGCTAGCGAACGTCTAGA
```